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ANALYSIS OF CORTISOL AND CORTICOSTERONE IN HUMAN PLASMA

by

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A THESIS

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The undersigned certify that they have read, and recommend
to the Faculty of Graduate Studies for acceptance, a thesis entitled:
"ANALYSIS OF CORTISOL AND CORTICOSTERONE IN HUMAN PLASMA", submitted
by Dorothy A. Jeffery, in partial fulfilment of the requirements for
the degree of Master of Science.

ABSTRACT

The analysis of cortisol and corticosterone in human plasma was investigated by the use of a fluorometric assay and a competitive protein binding radioassay.

The two steroids were made to fluoresce by treatment with sulfuric acid in ethanol. Fluorometric assay of the two steroids individually was done by establishing that known concentrations of the pure steroids would give reliable standard curves. Since both cortisol and corticosterone fluoresce under the same conditions, it was necessary to establish a satisfactory means to resolve the two. The method of Matsumara (39), which is based on different relative fluorescence of cortisol and corticosterone in different strengths of acid, was first employed. It was found that this method did not give satisfactory results. Following this, attempts were made to physically separate cortisol and corticosterone from mixed samples of the two steroids, so that the individual steroids could be measured fluorometrically. Carbon tetrachloride/water partitioning was used and found to give adequate separation for individual assay of cortisol. The interference of cholesterol, which fluoresces to a small but significant extent when its large concentration in plasma is considered, was not eliminated by solvent partitioning. Coupling of several solvent partitioning steps caused severe losses of corticosteroids; thus the fluorometric assay could not be applied to plasma samples.

For the competitive protein binding radioassay proper conditions for assay of samples of steroid which were of known concentration and which were known to be pure, were determined. When an adequate assay had been established, it was tested for specificity in detecting
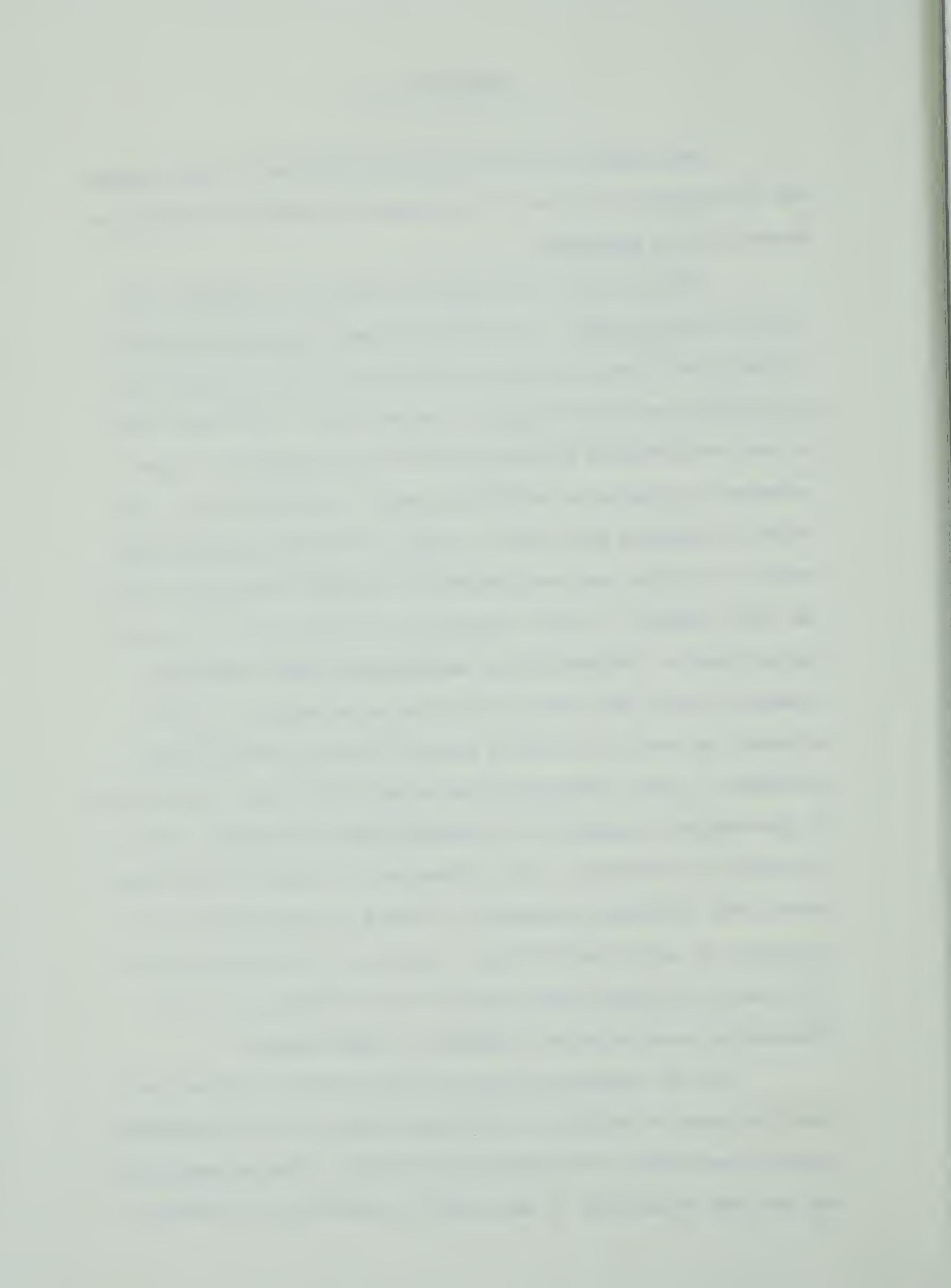
cortisol and corticosterone individually. Since cortisol and corticosterone were detected to approximately an equal extent, some separation of the two was needed. The carbon tetrachloride/water partitioning method seemed adequate. Next, it was necessary to prepare the plasma samples in a form acceptable for the radioassay. This consisted of removal of specific binding protein from the plasma by ethanol precipitation. When the means of recovery from plasma, means of separation and means of assay were established, they were used together as a total assay method. This total method, from the point of view of specificity, precision and accuracy, was satisfactory for the assay of cortisol in human plasma. The method, on the other hand, was not as reliable for the determination of corticosterone.

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Allodihydro-cortisol	5α-Pregnane-11β,17α,21-triol-3,20-dione
Androstenedione	Androst-4-ene-3,17-dione
Androsterone	5α-Androstan-3α-ol-17-one
Betamethasone	9-Fluoro-16β-methylpregn-1,4-diene-11β,17,21-triol-3,20-dione
Cholesterol (Chol)	Cholest-5-en-3β-ol
Corticosterone (B*)	Pregn-4-ene-11β,21-diol-3,20-dione
Cortisol (Hydro-cortisone, F*)	Pregn-4-ene-11β,17α,21-triol-3,20-dione
Cortisone (E*)	Pregn-4-ene-17α,21-diol-3,11,20-trione
11-Dehydrocorti-costerone (A*)	Pregn-4-en-21-ol-3,11,20-trione
21-Dehydrocortisol	Pregn-4-en-21-al-11β,17α-diol-3,20-dione
Dehydroepiandro-sterone (DHEA)	Androst-5-en-3β-ol-17-one
11-Deoxycorti-costerone (Cortexone, DOC)	Pregn-4-en-21-ol-3,20-dione
11-Deoxycortisol (Cortexolone, S**)	Pregn-4-ene-17α,21-diol-3,20-dione
21-Deoxycortisol (11,17-Dihydroxy-progesterone)	Pregn-4-ene-11β,17α-diol-3,20-dione
Dexamethasone	9α-Fluoro-16α-methylpregn-1,4-diene-11β,17α,21-triol-3,20-dione
Estradiol	Estra-1,3,5-triene-3,17β-diol
Estriol	Estra-1,3,5-triene-3,16α,17β-triol
Estrone	Estra-1,3,5-trien-3-ol-17-one

List of Steroid Names (continued)

17α-Ethinyl-testosterone	17α-Ethinylandrost-4-en-17β-ol-3-one
Etiocholanolone	5β-Androstan-3α-ol-17-one
11-Hydroxy-androstenedione	Androst-4-en-11β-ol-3,17-dione
20-Hydroxycortisol (Reichstein's substance E)	Pregn-4-ene-11β,17α,20β,21-tetrol-3-one
18-Hydroxydeoxy-corticosterone	Pregn-4-ene-18,21-diol-3,20-dione
11-Hydroxy-progesterone	Pregn-4-en-11β-ol-3,20-dione
17-Hydroxy-progesterone	Pregn-4-en-17α-ol-3,20-dione
Methylprednisolone	6α-Methylpregn-1,4-diene-11β,17α,21-triol-3,20-dione
Methyltestosterone	17α-Methylandrost-4-en-11β-ol-3-one
Paramethasone	6α-Fluoro-16α-methylpregn-1,4-diene-11β,17α,21-triol-3,20-dione
Prednisolone	Pregn-1,4-diene-11β,17α,21-triol-3,20-dione
Prednisone	Pregn-1,4-diene-17α,21-diol-3,11,21-trione
Pregnanediol	5β-Pregnane-3α,20-diol
Pregnanetriol	5β-Pregnane-3α,17α,20-triol
Pregnenolone	Pregn-5-en-3β-ol-20-one
Progesterone (P)	Pregn-4-ene-3,20-dione
Reichstein's substance U	Pregn-4-ene-17α,20β,21-triol-3,11-dione
Testosterone	Androst-4-en-17β-ol-3-one
Tetrahydro-cortisol (THF)	5β-Pregnane-3α,11β,17α,21-tetrol-20-one
Tetrahydro-cortisone (THE)	5β-Pregnane-3α,17α,21-triol-11,20-dione
Triamcinolone	9α-Fluoropregn-1,4-diene-11β,16α,17,21-tetrol-3,20-dione

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* Kendall's symbol.   ** Reichstein's symbol.

LIST OF ABBREVIATIONS

abs	absolute
ACTH	adrenocorticotropic hormone
AM	ante meridiem
b	slope
t-BuOH	tertiary butanol
C	Centigrade
^{14}C	Carbon-14
CBC	cortisol binding capacity
CBG	corticosteroid binding globulin, transcortin
cc	column chromatography
Ci	curie
cm	centimeter
CPB	competitive protein binding
CS	corticosteroids
CSF	cerebrospinal fluid
cv	coefficient of variability
ε	extinction coefficient
ecd	electron capture detector
EtOAc	ethyl acetate
EtOH	ethanol
fid	flame ionization detector
g	gram
gla	glacial
glc	gas liquid chromatography
GM	Geiger-Mueller
^{3}H, T	tritium, Hydrogen-3
hr, hrs	hour, hours

List of Abbreviations (continued)

^{125}I, ^{131}I	Iodine-125, Iodine-131
INH	isonicotinic acid hydrazide
17-KS	17-ketosteroids
M	molar
MeOH	methanol
meq	milliequivalent
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mM	millimole
mμ	millimicron
mμg	millimicrogram, nanogram
N	normal
11-OHCS	11-hydroxycorticosteroids
17-OHCS	17-hydroxycorticosteroids
pc	paper chromatography
pet ether	petroleum ether
PM	post meridiem
POPOP	1,4-di- [2(5-phenyloxazolyl)] -benzene
PPO	2,5-diphenyloxazol
P-S	Porter-Silber
rpm	revolutions per minute
s	standard deviation
^{35}S	Sulfur-35
se	standard error
sec	second(s)
tlc	thin layer chromatography

List of Abbreviations (continued)

TMS(E)	trimethylsilyl (ether)
µCi	microcurie
µg	microgram
µl	microlitre
U V	ultraviolet

Chemical formulae have been used for abbreviations in some tables.

Solvents for chromatography systems were abbreviated with the following letters:

A	acetone
Am	ammonium hydroxide
B	benzene
BA	butyl acetate
Bu	butanol
C	cyclohexane
Cf	chloroform
Ct	carbon tetrachloride
D	dioxane
DMF	dimethylformamide
E	ethanol
EA	ethyl acetate
EC	ethylene chloride
F	formamide
G	glycerol
H	hexane
HA	acetic acid
I	iso-octane

List of Abbreviations (continued)

IPE	isopropyl ether
L	ligroin
LP	light petroleum
M	methanol
MC	methylene chloride
Mes	mesitylene
PE	petroleum ether
PG	propylene glycol
T	toluene
W	water

INTRODUCTION

In recent years there has been considerable interest in the determination of adrenocortical steroids in peripheral plasma. The information derived is useful for clinical diagnosis and for investigation. The first methods developed determined groups of steroids which possessed common structural features (eg. 17-ketosteroids, 17-hydroxycorticosteroids), but later methods allowed the determination of specific steroids. Many approaches to the determination of adrenal steroids have been reported in the literature and several reviews on the subject have appeared (1, 2, 3). The methods reviewed in this study include absorption spectrophotometry, fluorometry, gas liquid chromatography, isotope dilution analysis and competitive protein binding radioassay. Chromatographic separation methods were reviewed and are presented as lists of systems in the appendix. Bioassay methods have not been considered, nor have the early formaldehydogenic steroid determinations. It was the purpose of this investigation to examine some of these methods with a view to finding a procedure which would be individually specific for cortisol and corticosterone, but at the same time be simple, rapid and reliable. Our concern was with unconjugated steroids in plasma since the conjugated metabolites are not biologically active.

SURVEY OF LITERATURE

A.   Spectrophotometric Methods.

Quantitation of steroids by absorption spectrophotometry can be achieved with various reagents. Alcoholic solutions of steroids containing the Δ^4-3-keto group (Figure 1) all absorb ultraviolet light in the region 240-242 mμ (molar extinction coefficient, $\mathcal{E} \approx 16000$) and at 310 m$\mu$ ($\mathcal{E} \approx 100$). The latter absorption band being weak is rarely reported in the literature. No specificity of quantitation can be achieved by measuring ultraviolet light absorption without prior separation of the steroids by some means (eg. tlc, pc, cc) (4). Ultraviolet ab-

Figure 1.   Structure of a Δ^4-3-Ketosteroid (Δ^4-3-Keto group is circled).

sorption can be used as a means of quantitation, but removal of impurities from the sample is very important since they may absorb light in the area of 240 mμ giving erroneously high results(5). For work with steroids in biological samples the sensitivity of ultraviolet assay is low, thus to estimate corticosterone in human blood a sample of 100 ml or more would be required. Cortisol has been determined in 40 ml of plasma by reading the absorbance at 240 mμ following chloroform extraction, Florisil chromatography, and paper chromatography (6).

Treatment of steroids with concentrated sulfuric acid for approximately two hours produces an absorption spectrum different from that of the native steroid. Some specificity is introduced because the products absorb at different wavelengths as can be seen in Table I (7).

TABLE I.

Absorption Maxima (mμ) of Some Steroids in Concentrated Sulfuric Acid.

Aldosterone		287-290		390	420	450
Corticosterone	241	280-288	320-331	370-375	418	440-460
Cortisol	237-240	278-285		380-395		470-480
Cholesterol	242		319		415	489

Concentrations of 5-50 μg/ml of sulfuric acid are used. If this were ap-
plied to quantitation of cortisol, an abundant corticosteroid, 50-500 ml
of blood plasma would be needed for detection with ordinary cuvettes.
The use of microcuvettes reduces the size of sample needed. Concentrated
sulfuric acid will cause charring of impurities in the sample giving rise
to interfering absorption, hence the sample must be pure before the ad-
dition of acid (7).

The α-ketol group (Figure 2) or a ring ketol in a steroid mol-
ecule possesses reducing ability(3,5). This group is present in cortisol,
corticosterone, cortisone, 11-deoxycortisol,
and 11-deoxycorticosterone. In the presence
of alkali the α-ketol group will reduce blue
tetrazolium (3,3⁻dianisole-bis [4,4⁻(3,5-
diphenyl) tetrazolium chloride]) to an insol-
uble blue colored formazone (3) which absorbs

Figure 2. Corticosterone
showing α-ketol group
(circled).

light at 510 mμ (5). The method is sensitive to 10 μg/ml of cortisone
acetate (8). Time for development of maximum color varies from one
steroid to another (9, 10). At room temperature, using sodium hydroxide
as the base and reading at 525 mμ, aldosterone developed maximum color
in 12 minutes, cortisone and deoxycorticosterone in 30 minutes, and corti-
costerone and cortisol in 50 minutes. Recknagel (9) studied various con-
ditions of the color reaction: type of base, rate of reaction, effect of

temperature, effect of light and effect of water. He found that water
inhibited the development of maximum color. Contrary to other workers,
he did not find that the reaction was sensitive to light. The blue tetra-
zolium reaction has been used to determine plasma corticoids (11, 12, 13,
14). Chen extracted 10 ml of plasma with methylene chloride, then washed
the extract with dilute sodium hydroxide and then water by a countercur-
rent method. Paper chromatography and washing with hexane/methanol were
the final purifications to precede elution and color development. Normal
corticoid levels were found to be 33.2 ± 8.27 (s) µg/100 ml. Recovery in
terms of cortisol was 85% ± 5. Hamman (15) used blue tetrazolium for
direct quantitation of corticoids on thin layer chromatography plates
with accurate results down to 0.1 µg.

The Zimmerman reaction detects ketosteroids. Various colors
are produced depending on the position of the carbonyl group. The 17-oxo
group gives a violet color, the 3-oxo a bluish tone, the 20-oxo a reddish-
brown tone and the 6-oxo a yellow-orange color (16). The Zimmerman reagent
consists of metadinitrobenzene in ethanol and potassium hydroxide (17).
It is possible to use the Zimmerman reagent to quantitate corticosteroids
if they are first oxidized to their corresponding 17-ketosteroid (18).

In 1950 Porter and Silber (19) reported a color reaction for 17,
21-dihydroxy-20-ketosteroids. Phenylhydrazine in sulfuric acid was re-
acted with the dihydroxyacetone group (Figure 3) to produce a chromogen
with an absorption maximum at 410 mµ. Presumably
a Δ^{16}-osazone was formed in the reaction by the
loss of water from carbons 16 and 17 (20). The
reagent reacted with cortisone, cortisone acetate,
11-deoxycortisol acetate, cortisol, tetrahydro-
cortisol, tetrahydrocortisone and 11-deoxycortisol (19, 21, 22).
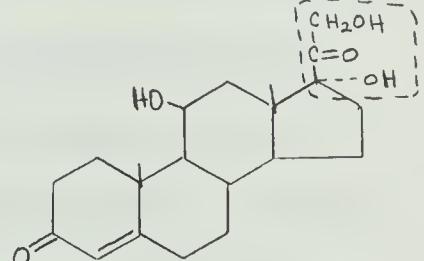
Figure 3. Cortisol showing dihydroxyacetone group (circled).

Steroids which produced less than 3% absorbance relative to cortisone were deoxycorticosterone acetate, corticosterone, 11-dehydrocorticosterone acetate, methyltestosterone, estradiol, 17-hydroxyprogesterone, androstenedione and androstenetrione. All these steroids lacked the 17, 21-di-hydroxy-20-keto group. Known interfering substances include fructose, dehydroascorbic acid, acetone, paraldehyde, bile, chlorpromazine, diethyl-aminoethyl phenothiazine and quinine (19, 20, 21, 23, 24). Heparin is preferred as the anticoagulant in plasma samples since citrate may give rise to cloudiness in the final solution (25). A large number of pro-cedures for determining 17-hydroxycorticosteroids using the Porter-Silber reagent have been developed. They are briefly summarized in Table XXXI in the appendix. Procedures appear in alphabetical order according to the principal author. Since the Porter-Silber reagent does not react with corticosterone this fact can be utilized in the determination of corticosterone. Corticosteroids are determined by a method which detects both cortisol and corticosterone (eg. blue tetrazolium or fluorometry) and also by the Porter-Silber reaction. The difference between the two values is attributed to corticosterone (26, 27).

Corticosteroids can be determined using 2,4-dinitrophenyl-hydrazine (28). This reagent has different specificity compared with phenylhydrazine. It detects cortisone, cortisol acetate, 11-deoxycort-isol, deoxycorticosterone, progesterone, testosterone, methyltestosterone and 17 α-ethinyltestosterone; but not androsterone, epiandrosterone, estriol or estrone. This reagent is used in acidic methanol solution. If the reaction is allowed to proceed for five minutes at 20°C Δ^4-3-keto-steroids react quantitatively while little interference is derived from other ketones. If the reaction is allowed to continue longer and at a higher temperature (90 minutes at 59°C) a carbon-20 keto group associated

with a dihydroxyacetone side chain reacts quantitatively in addition to the Δ^4-3-keto group, while a carbon-20 ketone associated with a monohydroxyacetone group reacts partially. When the reaction was used for the detection of plasma corticosteroids in patients being given ACTH infusions, 10 ml of sample were extracted three times with 75% alcohol, the supernatant was washed with petroleum ether and the ethanol phase was concentrated to a volume of 20 ml. This was then extracted with chloroform and the extract was washed with 5% sodium carbonate solution. After partitioning between benzene and water, the water was evaporated and the residue dissolved in methanol. The 2,4-dinitrophenylhydrazine reagent was added and the mixture was incubated and the absorption finally read at 275 mμ. For cortisone a plot of mass versus absorbance was linear over the range 0 to 20 μg/0.5 ml.

The reaction of isonicotinic acid hydrazide (INH) in acidified alcohol with α, β -unsaturated ketones to produce a yellow hydrazone (λ max 380 mμ) has been used to provide a color reaction for Δ^4-3-ketosteroids (29). Because most biologically active corticosteroids contain this group, the method appeared useful for the assay of corticoid activity. INH will not react with saturated ketosteroids or with ketosteroids where the double bond is not in an α,β -position; thus it does not react with allodihydrocortisol or other reduced cortisol metabolites. For plasma determinations at least 20 ml of sample were required. The plasma was extracted and the extract purified by column chromatography. The standard curve covered the range 0.5 to 8 μg/0.5 ml. Normal values of 13.3 ± 1.05 (se) μg/100 ml were found. Because of the specificity for α,β -unsaturated ketones INH values of corticosteroids are lower than values determined by the Porter-Silber or blue tetrazolium methods.

The Allen correction method has been used in many described

methods. It is a mathematical technique which permits the analysis of absorption curves produced by a mixture of substances provided the absorption curve of the contaminant approximates a straight line (30). Two wavelengths, a and b, equidistant on either side of the absorption maximum, x, are chosen and readings are taken at all three wavelengths. The formula: $CD_X = OD_X - \dfrac{OD_a + OD_b}{2}$ is used to determine the absorbance, CD_X, of the unknown, X, without the contribution of the contaminant. This method is valid only if the absorption curve of the contaminant is linear; in many cases this has not been determined and it may not be safe to assume this linearity.

B.   Fluorometric Methods.

Corticosteroids do not possess native fluorescence but can be made to fluoresce by treatment with strong acid. The resulting fluorophors are activated at 470-475 mμ and fluoresce at 520-530 mμ. The chemical nature of the fluorescent species is not known.

Since methods of analysis have been derived empirically, many slight variations are found. Although many authors omit steps for simplicity or add steps to improve specificity, the basic procedure for the fluorometric determination of corticosteroids in plasma is as follows.

(1) Washing of plasma with petroleum ether or iso-octane to remove neutral lipids or sterols.

(2) Dilution of plasma with alkali to fix acidic materials, notably estrogens, which possess a phenolic hydroxyl group in the A ring. Alternately the plasma extract may be washed with alkali to rid it of acidic material. In this case the extract must be dried with anhydrous sodium sulfate or calcium chloride. Stewart (31) preferred the first procedure because drying agents increased the background

fluorescence of the samples. Dilution of the plasma with alkali gave clearer extracts which produced higher fluorescence. Destruction of steroids by alkali was prevented due to the short time of exposure and also due to the buffering action of the plasma proteins.

(3) Extraction of the plasma with ethyl acetate, methylene chloride, chloroform, carbon tetrachloride or a mixture of these solvents to obtain the corticosteroids.

(4) Evaporation of extraction solvent and addition of acid fluorescence reagent or extraction of steroids from the solvent into the acid reagent and aspiration of the solvent layer. Development time varies with the various procedures. Table XXXII in the appendix summarizes fluorometric procedures for the determination of corticoids. Methods are classified according to the strength of the acid reagent and within these groups arranged alphabetically.

Potentiation or quenching of fluorescence may result from small amounts of impurities (32). One important problem with regard to fluorometric assay is that of interfering fluorescence. This may arise from glassware, solvents and reagents or the sample itself. For this reason glassware requires special cleaning precautions. Many synthetic detergents are fluorescent so they should not be used for cleaning of glassware. To avoid this problem an inorganic soap, such as Calgonite, should be used (33). If chromic acid is used it must be carefully removed, since even slight traces will absorb ultraviolet light. Chromic acid should not be used to wash cuvettes; nitric acid is preferred (33). Martin (34) soaked all glassware in nitric acid overnight, then rinsed it thoroughly (four hours) with tap and distilled water.

Purification of solvents and reagents is very important in order to lower background fluorescence. Undenfriend (33) reported that butanol,

ether, ethylene dichloride, benzene and heptane could be purified for fluorometry by washing successively with 0.1 N hydrochloric acid, 0.1 N sodium hydroxide and water. Further purification if necessary, could be achieved by passage over silica gel. Absolute ethanol should be distilled from potassium hydroxide for fluorometry near the limits of detection. Fisher (35) showed that the addition of a long chain hydrocarbon (eg. paraffin) or a long chain alcohol (eg. cetyl alcohol) prior to distillation gave solvents with lower fluorescence compared to solvents distilled alone.

Chemical structures responsible for acid-induced fluorescence by steroids are 11-hydroxyl and Δ^4-3-keto groups (36). Steroids possessing these structures include cortisol, corticosterone, 20 β -hydroxycortisol and their 11-hydroxy epimers (37). Fluorescence of Δ^4-steroids is increased by the presence of a 17- or 21-hydroxyl group. On the other hand fluorescence was found to be reduced by the introduction of an 11-keto or 16-hydroxyl group or by reduction of the double bond at the 4 position (38). Table II shows the relative fluorescence of several steroids in various strengths of acid. Substances which have been tested and found to be non-fluorescent are shown in Table III. Cholesterol, although very low in fluorescence, is present in the blood in very high concentration relative to other steroids and thus produces a severe interference problem (39). Flack (46) presented a method to mathematically eliminate the contribution of cholesterol using orthogonal polynomials and reading the fluorescence over the range 520-540 mμ rather than only one wavelength. The method is applicable to other interfering fluorogens.

Corticosteroids can also be made to fluoresce in the presence of antimony trichloride and acetic acid or by treatment with phosphoric acid (47). They also fluoresce under ultraviolet light when treated

with alkali. This property has been applied to the determination of corti-
coids in 10 ml of plasma. After separation by paper chromatography
cortisol concentration was estimated as 7-13 µg/100 ml and cortisone
1.5-5 µg/100 ml (48). Potassium t-butoxide has been used to quantitate
Δ^4-3-ketosteroids (49). The procedure was sensitive to 0.01 µg and
detected cortisol, corticosterone, cortisone, cortisone acetate, 11-
dehydrocorticosterone, deoxycorticosterone, progesterone and testosterone.

TABLE II.

Relative Fluorescence of Equal Amounts of Steroids in Various
Concentrations of Sulfuric Acid.

Steroid	Fluorescence Reagent (expressed as acid concentration)			
	60%[a]	70%[b]	75%[acd]	80%[efgh]
Corticosterone	1	1	1[ad]	1
Cortisol	0.265	0.3	0.884[a]	1[fg]
			0.95[c]	0.5[gh]
Cortisone	0	0.01	0.001[a]	0[gh]
Deoxycorti-corticosterone	-	0.01	0[d]	0.75[f]
				0[g]
				0.0094[h]
11-Deoxycortisol	-	0.01	-	-
Aldosterone	-	0	-	-
20β-Hydroxy-cortisol	-	-	0.87[c]	0.15[g]
21-Deoxycortisol	-	-	0.15[c]	-
Progesterone	0.003	-	0.003[a]	-
11β, 17α-Dihydroxy-progesterone	-	-	-	0.085[g]
Estradiol	0.565	-	3.30[a]	0.32[g]
Estrone	-	-	-	0.10[g]
Testosterone	0.07	-	0.1[a]	5[f]
Cholesterol	0.005	-	0.01[a]	0.0026[h]
Pregnanetriol	-	-	0.62*[d]	-
Pregnanetriolone	-	-	0.145*[d]	-
Prednisolone	-	-	-	0.05[h]

* mean of 1-4 μg of test compound compared to 0.2 μg of cortisol taken
  as 1.

[a] Refers to reference #39.
[b] Refers to reference #40.
[c] Refers to reference #37.
[d] Refers to reference #41.
[e] Refers to reference #42.
[f] Refers to reference #43.
[g] Refers to reference #44.
[h] Refers to reference #45.

TABLE III.

Substances Found Not to Possess Acid-Induced Fluorescence.

75% H_2SO_4[c]	80% H_2SO_4[g]
-	Cortisone
Deoxycorticosterone	Deoxycorticosterone
11-Deoxycortisol	11-Deoxycortisol
-	11-Dehydrocorticosterone
-	Reichsteins U
-	Tetrahydrocortisone
-	Tetrahydrocortisol
-	Allodihydrocortisol
-	9 α -Fluorocortisol
Prednisone	Prednisone
Prednisolone	Prednisolone
-	9 α -Fluoroprednisolone
Dexamethasone	-
Progesterone	Progesterone
11-Hydroxyprogesterone	11 β -Hydroxyprogesterone
-	Testosterone
Dehydroepiandrosterone	-
11-Hydroxyandrosterone	-
11-Hydroxyetiocholanolone	-
Androstenedione	-
-	Allodihydrotesterone
Glucose	-
Fructose	-
Acetone	-
β -Oxybutyric acid	-

C.   Gas Liquid Chromatography Methods.

Gas liquid chromatography (glc) is a useful technique for the analysis of complex mixtures because it affords a means of separation and quantitation in one procedure. The use of glc with steroids requires columns with low concentrations of stationary phase. Determination of steroids in biological samples (eg. plasma, urine, tissue) necessitates the use of detectors with high sensitivities because of the very low concentration of steroids present. The ionization and electron capture detectors are examples of high sensitivity detectors.

Gas chromatography of corticosteroids has been attempted using the steroid without derivative formation. This approach avoids problems which could arise from non-quantitative conversion of steroids to derivatives (50); however, another problem is encountered because as the number of oxygen functions in a steroid molecule increases so does its retention time and its susceptibility to decomposition (51). Corticosteroids have a large number of oxygen functions, therefore they will have long retention times and are likely to breakdown when subjected to glc. Vandenheuvel (51) found that 20-one-17α,21-diol steroids (eg. cortisol, cortisone, 11-deoxycortisol) gave single peaks when chromatographed on 1.5% SE-30 at 222°C using an argon ionization detector. Retention times were 0.91, 0.65, and 0.54 respectively relative to cholestane = 1 (where 1=12.6 minutes). The peaks corresponded exactly to their respective 17-keto-4-androstene analogs. Investigation suggested that the loss of the

Cortisol   Androst-4-en-11β-ol-3,17-dione
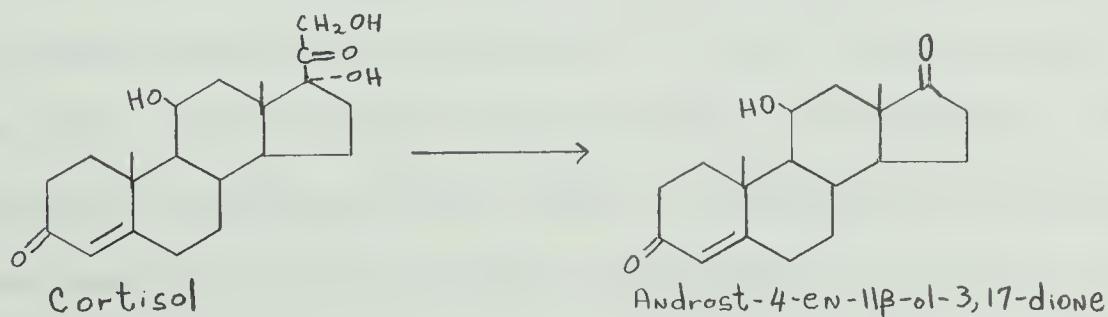
Figure 4. Decomposition of Cortisol to a Corresponding 17-Ketosteroid.

side chain (Figure 4) occurred during the vaporization stage in the "flash heater" and not on the column since no tailing, which is characteristic of on-column decomposition, was observed. Steroids lacking the 17 α -hydroxyl (eg. corticosterone, deoxycorticosterone) did not decompose in this manner; nevertheless they did degrade during gas chromatography. Corticosterone gave rise to four major peaks and deoxycorticosterone to two major peaks. Neither aldosterone nor its 21-acetate could be chromatographed under the conditions used by Vandenheuvel. Gottfried (52) using a 3.8% SE-30 column at 240°C, found that cortisol gave a linear plot of mass injected versus peak area for the 17-ketosteroid produced over the range 75-1500 nano-grams when using a flame ionization detector. Cortisone and 11-deoxy-cortisol were not found to give linear graphs. Differences in the slopes of the three graphs and their non-linearity was attributed to incomplete conversion of the corticosteroids to 17-ketosteroids; however, the con-tribution of irreversible adsorption to the column was not ruled out. Crane (50) quantitated urinary corticoids by gas chromatography using 5% SE-30 at 253°C and a radium or strontium-90 ionization detector. Cortisol had a retention time of 318 seconds. Urine was prepared for assay by acid hydrolysis at pH 1, chloroform extraction, washing with 0.1 N NaOH and two paper chromatographies. Equal weights of different steroids were found to give different peak areas when chromatographed even though retention times were similar. Adrenal vein blood levels of 17-hydroxycorticoids, notably cortisol and 11-deoxycortisol, were deter-mined by gas chromatography on 1.5% SE-52 at 210°C using a strontium-90 detector (53). Cortisol had a retention time of 24.8 minutes. Since pyrolysis of 17-hydroxycorticoids leads to formation of 17-ketosteroids, these two fractions in the blood were separated on a silicic acid micro-column prior to glc. Five milliliters of peripheral blood was extracted,

then the extract was fractionated on the micro-column. The more polar 17-hydroxycorticoids came off the column after the 17-ketosteroids. The eluates were evaporated, dissolved in methanol and injected into the gas chromatograph. Corticosterone and other 17-deoxycorticosteroids were not detected by this method.

Preparation of acetate derivatives of corticosteroids has been used to reduce the polarity of these compounds by masking the hydroxyl groups. Since the acetate derivatives are still subject to decomposition during gas chromatography, this attempt to improve glc characteristics has not been entirely successful. Aldosterone, as the alcohol, has been found to give rise to three peaks on glc which are variable and unsuitable for quantitation, so oxidation and acetylation were studied in an effort to find a suitable derivative (54). After chromate oxidation, the aldosterone γ-lactone produced, when chromatographed on 2% SE-30, gave a single peak which was suitable for quantitation. The 21-monoacetate derivative of aldosterone produced two peaks when chromatographed, while the 18,21-diacetate gave one peak suitable for quantitation. This peak arose from an unidentified degradation product of the diacetate. The limit of detection for this derivative, using an argon ionization detector, was 0.02 μg while the limit of quantitation was 0.1 μg. Wotiz (55) also observed a single symmetrical peak from glc of aldosterone 18,21-diacetate. Brooks (56, 57, 58) studied the degradation of acetate derivatives of 20-oxo-pregnene type of steroids. Cortisol, corticosterone, and aldosterone, although easily acetylated, were found to give chromatograms unsuitable for quantitative work when 1% SE-30 at 225°C was used. Since the corticosteroid acetates could be sublimed <u>in vacuo</u> at 200°C with only slight decomposition, Brooks felt that the decomposition during glc must be a catalytic process and not solely thermal degradation. Assays of acetylated urinary corticosteroids

by glc has been used to obtain qualitative and quantitative information
(59, 60).

Electron capture detectors increase the sensitivity of gas chrom-
atography. They are based on the ability of certain organic molecules to
capture electrons in the detector. This ability is possessed by highly
electro-negative compounds (eg. halogen containing compounds). Certain
steroids have been found to capture electrons (61). Rapp (62) investigated
glc of corticosteroid acetates and chloroacetates using an electron capture
detector and a 1% SE-30 column operated at 206°C. Generally, the acetate
derivatives were judged superior to the chloroacetates, since the detector
was less sensitive to the latter compounds. Corticosterone acetate was
quantitated to a lower limit of 15 nanograms. Cortisol acetate gave a
broad peak which was not suitable for quantitation.

Since the degradation of 17-hydroxycorticoids to 17-ketosteroids
occurring on glc may not be complete, some workers have oxidized the cort-
icoids prior to chromatography. Bailey (63) noticed an increase in sensi-
tivity with the initial oxidation to 17-ketosteroids. The latter investi-
gator also quantitated cortisol, cortisone, prednisone, and prednisolone
after bismuthate oxidation to corresponding 17-ketosteroids. Gas chroma-
tography was carried out on 0.65% neopentyl glycol adipate at 230°C. The
linear range of the peak area versus mass plot was 2 to 8 micrograms using
Strontium-90 ionization detector. Corticosteroids in urine were assayed
by glc subsequent to ethyl acetate extraction, paper chromatography in
Bush B_5 system and elution of cortisol and cortisone regions. These eluates
were oxidized. Prednisone in a known amount was added to the urine prior
to extraction to act as an internal standard. The use of a simple solid
injection system overcame the problem of taking up the whole extract in
one or two microlitres of solvent (63, 64). More recently, Van der
Molen (65) has shown glc to be feasible for the determination of sub-

microgram amounts of plasma steroids. Beale (66) explored various con-
ditions for use of sodium bismuthate as an oxidant and was able to find
conditions which allowed oxidation of urine extracts without pre-chrom-
atography. Periodate oxidation of 20,21-ketol steroids leads to produc-
tion of corresponding etiocholenic acids, except for aldosterone which is
converted to an internal ester (a lactone) (67, 68). These etiocholenic
acids were not suitable for gas chromatography so they were reacted with
diazomethane to form their methyl esters. The sequence of reaction for
preparation of derivatives suitable for glc is shown in Figure 5.

Corticosterone   an etiocholenic acid   17β-Carbomethoxy-
                                                          androst-4-en-11β-ol-3-one
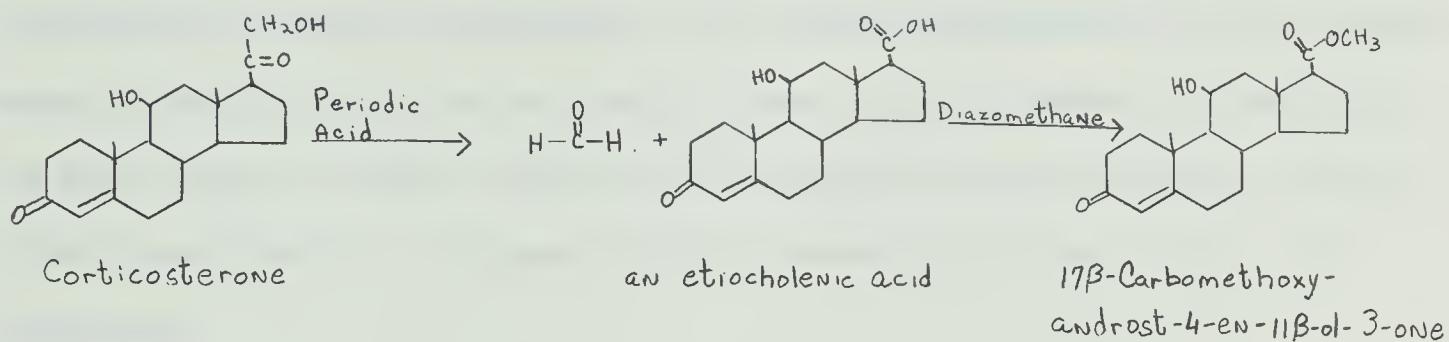
Figure 5. Reaction Sequence for Preparation of a
Corticosterone Derivative Suitable for glc.

Because aldosterone was oxidized to a lactone, which is non-acidic and
thus not soluble in sodium bicarbonate solution, the aldosterone deriv-
ative can be separated from the other corticoids at this point. Also,
because of this lack of a carboxyl group it can be chromatographed di-
rectly. Separation of the methyl esters of the etiocholenic acid deriv-
atives of corticosterone, deoxycorticosterone, deoxycortisol, cortisone
and cortisol was achieved at 220°C on 0.8% SE-30, 0.5% Pluronic 68 and
0.3% neopentyl glycol succinate (68). The latter stationary phase gave
the best separation. Kittinger (67) developed this technique further
and was able to use it quantitatively for several steroids including
corticosterone, aldosterone, deoxycorticosterone, and progesterone.
Gas liquid chromatographic analysis of both 17-deoxy- and 17 α-hydroxy-

corticoids was developed by combining these various derivatives (69). The initial steroid mixture was treated with periodate in aqueous dioxane producing etiocholenic acid derivatives from all 20,21-ketol steroids. These acidic derivatives were removed by extraction with aqueous alkali and then esterified with diazomethane. The neutral portion was treated with butyric anhydride in pyridine to acylate the 3- and 17 α-hydroxyl groups. These acylated derivatives can also be subjected to glc. Aldosterone, cholesterol, corticosterone, cortisol, cortisone, dehydrocorticosterone, dehydroepiandrosterone, deoxycorticosterone, 11-deoxycortisol, 11 β-hydroxyandrostenedione, 18-hydroxy-11-deoxycorticosterone, 11 β-hydroxyprogesterone, 17 α-hydroxyprogesterone, pregnenolone and progesterone were determined. Quantitation was done using a 1% QF-1 column at 260°C with an argon ionization detector (Strontium-90 cell). The lower limit of quantitation was 0.01 to 0.02 micrograms.

Efforts to stabilize the dihydroxyacetone group were made by reaction of cortisone with formaldehyde in acid media to give the bismethylenedioxy derivative (70). The resultant gas chromatogram was quite complex.

Trimethylsilyl ether (TMSE) derivatives of corticosteroids have been used in glc analysis because of their increased volatility relative to the initial steroid. In forming the TMSE derivatives unhindered hydroxyl groups are converted to ethers while ketones are left unchanged (71). Gottfried (72) prepared TMSE derivatives of aldosterone, cortisol, corticosterone, cortisone, deoxycorticosterone, and 11-dehydrocorticosterone. After thin layer chromatography purification, the derivatives were submitted to gas chromatography on 3.8% SE-30 at 275°C using a flame ionization detector. Corticosterone, cortisone, deoxycorticosterone, and 11-dehydrocorticosterone TMSE derivatives exhibited poor thermal stability but if reduced at the 20-hydroxyl position prior to TMSE formation, more

stable derivatives were obtained. All trimethylsilyl ether derivatives, except cortisol, gave single peaks. The lower limit of sensitivity was 25 millimicrograms. Since trimethylsilyl ethers are susceptible to hydrolysis by moisture their handling must be rapid. Gottfried did apply this glc method to the determination of cortisol in hamster adrenal vein blood.

D.   Isotope Derivative Methods.

Double isotope derivative methods for the determination of steroids offer very great sensitivity compared to other methods and for this reason can be employed to measure plasma aldosterone, the concentration of which is in the mμg/100 ml range. There are two basic approaches to isotope derivative analysis: the double isotope derivative dilution method (73) (also referred to as the complete indicator method) (74), and the double isotope derivative method (partial indicator method). In the double isotope derivative dilution method, the radioactive indicator of recovery is added to the initial sample, while in the double isotope derivative method the labelled recovery indicator is added after preparation of the radioactive derivative of the compound of interest. In the latter method recovery prior to derivative formation should be complete and reproducible. The general principle of the method is as follows. A derivative of the steroid to be quantitated is prepared using a labelled reagent of known specific activity. The derivative is then rigorously purified. Knowing the stoichiometric equivalents of the steroid and the reagent, the quantity of steroid initially present in the sample can be determined from the activity of the pure derivative and the specific activity of the reagent. The rigorous purification procedure leads to loss of derivative so a radioactive recovery indicator labelled

with a different isotope than the reagent, is added. This indicator is
either the steroid being determined in the complete indicator method, or
the steroid derivative in the partial indicator method. The indicator
also serves as a measure of purity and identity. The principle involved
in establishing identity is that if an unknown radioactive molecule and a
known radioactive molecule (different label) can be purified to a constant
activity ratio the two molecules can be considered to be identical (73).
Constancy of isotope ratio is also accepted as a measure of purity since
if there is a radioactive impurity present repeated purification would
lead to a disproportionate loss of activity of one of the isotopes. This
means of establishing purity is not unequivocal since in a very complex
steroid mixture the chance of obtaining a constant ratio without having
achieved purity is not entirely negligible (73). Purity of a chromato-
graphic spot is established by demonstrating homogeneous isotope ratio
throughout the spot. Isotope fractionation, separation of one isotope
from another, has been observed with ^{3}H-aldosterone. This will result
in an inhomogeneous spot even when purity has been achieved. If frac-
tionation does occur, complete elution of a chromatographic spot is very
important in order to avoid loss of one isotopic form of the steroid re-
lative to the other (75). Purification is usually achieved by a series
of chromatographies and chemical conversions. The rationale of chemical
conversion is that although the steroid derivative and impurity may in-
itially have the same chromatographic mobility, it is unlikely that they
will have the same mobilities after chemical transformation (74). The
sensitivity of the method is limited by the specific activity of the label-
ling reagent and by the counting time available. Isotopes which have been
used in double isotope analysis of steroids are tritium, Carbon-14, Sulfur-
35, Iodine-125, and Iodine-131 (74). Sensitivity is also dependent on the

noise level, which is composed of statistical counting error and chemical noise. The former depends on the quality of the counting equipment and the latter, which is often more important, depends on radiochemical purity and the occurrence of exchange reactions (74).

Table XXXIII in the appendix summarizes some double isotope analyses of corticosteroids. Partial indicator methods are listed first followed by complete indicator methods.

An isotope method for the determination of aldosterone, corticosterone and cortisol using ^{14}C-blue tetrazolium has been reported (76). After chromatography on methanol, sodium hydroxide washed papers to separate the individual compounds, the steroids were reacted with alkaline ^{14}C-blue tetrazolium. The reaction was stopped with 2% acetic acid and excess reagent was removed by soaking in this solution. The radioactive derivatives were then eluted and counted. A standard curve was prepared covering the range 0.01 to 5 micrograms. The lower limit of sensitivity was 0.03 micrograms. This is approximately ten times better than the blue tetrazolium color reaction, but not as good as double isotope derivative methods. This approach has been used to determine plasma glucocorticoids using different conditions and using tritiated tetrazolium (77).

E. Competitive Protein Binding Radioassay Methods.

1. Characteristics of Corticosteroid Binding Globulin.

Transcortin, an alpha-globulin and glycoprotein, has a high affinity for binding corticosteroids. It is also referred to as corticosteroid-binding globulin (CBG). The molecular weight of human CBG has been reported as 40,000 (78), 45,000 (79), 51,700 (80), 52,000 (81), and 58,500 (82).

The interaction of CBG with steroids can be studied by equilibrium dialysis (83), gel filtration (84), ultrafiltration (85), fluorescence quenching (86), ultraviolet absorption (87), and competitive adsorption (88). A number of properties of interest with regard to this interaction is the capacity of the protein to interact with a particular steroid, the affinity of the protein for a steroid, the number of binding sites available, and the nature of the steroid protein interactions. DeMoor (89) has defined binding capacity as a measure of the number of binding sites for the steroid, and further described binding affinity or association constant as an indication of the strength of binding.

Cortisol binding capacity of human plasma has been reported as approximately 17.6 μg/100 ml (10-37°C) (90), 22 μg/100 ml (4°C) (91), 23 μg/100 ml (37°C) (92), 24 μg/100 ml (20°C) (89), and 31 μg/100 ml (9°C) (93). This represents a low binding capacity.

Cortisol binding affinity on the other hand is high. DeMoor (89) reported the cortisol binding affinity of human plasma to be 7.4 x 10^7 M^{-1} (20°C). Other workers reported 3.2 x 10^8 M^{-1} (37°C) (94) and 10 x 10^7 M^{-1} (37°C) (92). The binding affinity of purified human CBG has been reported as 2.4 x 10^7 M^{-1} (37°C) and 5.2 x 10^8 M^{-1} (4°C) (80).

The nature of the steroid-transcortin interaction is not known. It is possible that the interaction involves hydrogen bonding, covalent bonding, polar interaction, or Van der Waals forces (87, 95).

Many factors affect CBG steroid interaction. One of these is temperature. Murphy (93) found the cortisol binding capacity at 37.5°C to be 9% of the binding capacity at 9°C; however, Goldie (90) observed that the change in binding capacity from 37° to 9°C was not significant. Cortisol binding affinity definitely does increase with a decrease in temperature, being strongest at 2°C and almost absent at 42°C (78).

Beisel (96) reported a twofold increase in cortisol binding affinity when the temperature was decreased from 37°C to 4°C, Goldie (90) reports a four-fold increase with a temperature drop from 37°C to 10°C. Westphal (97) and Muldoon (80) observed a twentyfold increase in association constant when the temperature was decreased from 37°C to 4°C. Neither Goldie (90) nor Westphal (97) have found any change in the number of binding sites on CBG when the temperature was decreased from 37°C to 10°C or 4°C; hence, the increase in cortisol binding affinity could not be explained on this basis. Association of cortisol with transcortin is very rapid; however the rate of dissociation is much slower and can be studied from the point of view of temperature effect. At near saturation the half dissociation time is 25 minutes at 4°C and 10 seconds at 37°C (98); thus the change in cortisol binding affinity with temperature may be due to a change in the rate of dissociation. Sufficiently high temperature will inactivate CBG. Heating to 60°C for 15 minutes will inactivate the cortisol binding activity to the extent of 50% (91, 94). Changes in temperature from 2°C to 42°C cause reversible alterations in the binding characteristics of trans-cortin (78).

Hydrogen ion concentration has only a slight effect on protein binding in the range attained _in vivo_ (96). There was a slight increase in the binding of cortisol by CBG at higher pH ranges. Transcortin was inactivated by storage below pH 5 for 24 hours at 4°C; neutralization did not restore the binding properties (94). Ohtsuka (99) reported that acidification to pH 4 decreased the binding of cortisol to CBG to 5% of the original binding, but had much less effect on the binding of a cort-isol degradation product (21-dehydrocortisol) reducing it by 50%. The binding of cortisol to transcortin was found to be relatively stable from pH 5 to 7.5, to increase somewhat at pH 8 and to fall markedly at pH 4 (99).

Dilution of plasma did not significantly affect cortisol bind-
ing capacity (93). It did make differences in the binding of various
steroids to CBG more pronounced, and also hastened equilibration of ster-
oids and CBG (96).

The chemical structure of a steroid affects its binding to CBG.
Human CBG obeys the "polarity rule" (95) at 37°C in that increasing the
number of polar groups on the steroid molecule causes a decrease in the
strength of binding (100). Functional groups necessary for the binding
of a steroid to CBG (Figure 6) are Δ^4-3-keto, at least two hydroxyls
at either 11 β -, 17 α -, or 21-position and the 20-keto group (96, 101).
The C-11 position is important in that introduction of a ketone or change
of configuration to 11 α -hydroxyl decreases binding (94, 96, 101).
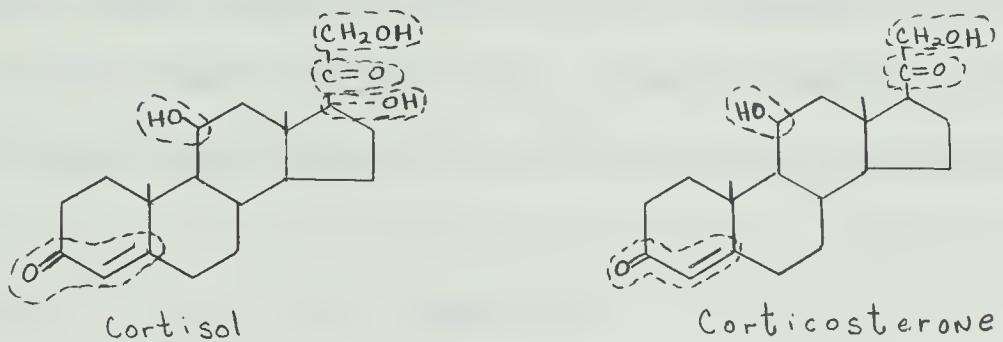
Figure 6. Structure of Steroids Bound to CBG.
(Circled functional groups are important to binding)

Glucuronide metabolites, which are more polar than the parent steroid,
have a low affinity for CBG (102); however they are bound to other plasma
proteins (103). Tetrahydro- and dihydro-metabolites of steroids have
been found to exhibit little binding to CBG (96). Unsaturation at carbon
1-2; 2-, 6-, or 16-methyl; 9 α -fluoro; 11 α -hydroxyl and 18-aldehyde
groups all decreased the binding of steroids to CBG (96, 101). This
fact may in part account for the increase in physiological activity of
steroids with these modifications in structure (96).

Species differences occur in the composition and binding char-
acteristics of transcortin. Westphal (97) ranked the concentration of CBG
binding sites for cortisol, corticosterone and progesterone as rat > monkey >
man > guinea pig > rabbit; the concentration of sites in rat being three
times that in rabbit. Binding affinity, as measured by the association
constant, varies with species and with steroid. At $37^\circ C$ the relative as-
sociation constants for cortisol are rabbit > man = monkey > rat > guinea pig,
while for corticosterone the order is monkey > man = rat > rabbit > guinea pig.

Protein binding of steroids is of interest from two points of
view: its biological significance and its usefulness for assaying very
small quantities of steroid hormones. The cortisol in blood is about 90%
protein bound (84, 93, 94, 98). The amount actually bound to CBG is con-
troversial; Murphy reports about 20% (93), Daughday 68.5% (94). Both
values are at normal cortisol levels. Table IV depicts the binding of
cortisol and corticosterone to the various protein fractions of human
serum.

TABLE IV.

Binding of Cortisol and Corticosterone to Blood Proteins.[a]

Blood Fraction	% Bound	
	Cortisol	Corticosterone
Alpha globulin (includes CBG)	6.4	2.2
Beta globulin	7.0	1.4
Gamma globulin	9.8	0.7
Albumin	58.6	84.4
Total protein bound	81.8	88.7
Unbound	18.2	11.3

[a] At $25^\circ C$ and pH 7.6 (95).

Because protein binding is reversible, it provides a circulating
reservoir of corticosteroid which is biologically inactive but available

when needed. The binding also prevents metabolism at the liver, filtration at the kidney, and perhaps protects steroids from chemical attack. Protein binding of steroids thus serves both a transport and a reservoir role. Protein binding of steroids is also very important because it is likely that one of the initial phases of action of most hormones must involve an interaction with a protein either in a target tissue or as part of an enzyme system (95, 96, 104).

    2. <u>Radioassay Using Corticosteroid Binding Globulin</u>.

Because corticosteroids make up a relatively limited group of compounds which are bound by CBG it is possible to assay corticosteroids using the property of protein binding. Furthermore different affinities of CBG for various corticosteroids afford a measure of specificity within this group. Several assays of corticosteroids based on protein binding have been reported (101, 105, 106, 107, 108, 109, 110). The basic principle involved in assay of corticosteroids by the method, named "competitive protein binding radioassay" by Murphy (111), is that CBG can be labelled to a certain specific activity with a radioactive steroid by equilibration of endogenous steroid on CBG with tracer steroid. Addition of further non-radioactive steroid will displace some of the CBG-bound radioactive steroid reducing the specific activity of the CBG in proportion to the quantity of steroid added. In order to perform an assay of a particular corticosteroid one needs a source of CBG which has a high affinity and specificity for the desired steroid, a radioisotope of the desired steroid, and a means of separating protein bound and unbound steroid. Table XXXIV in the appendix summarizes the competitive protein binding assays for corticosteroids by various workers.

In the assay a standard curve is prepared using various amounts of the desired steroid. The samples and standards are equilibrated with

CBG isotope solution, then the protein-bound and unbound steroid fractions are separated. The radioactivity of the protein-bound steroid is usually determined and plotted against the amount of steroid in the standards. The lesser the number of counts in the protein-bound fraction, the greater the amount of steroid present in the sample. If the time to accumulate a given number of counts is plotted versus the weight of steroid in the standards a linear graph with a positive slope will result. The weight of steroid in the samples can be determined from the standard curve.

Considerable testing of the specificity of this type of method has been done as shown in Table V.

Other steroids are now being assayed by protein binding radioassay. Kato (112), Horton (113), Hallberg (114), Mayes (115), Maeda (116) and Pizarro (117) have reported methods to assay testosterone using testosterone binding globulin, which is found in late pregnancy plasma. This protein is a beta globulin having a high affinity and low capacity for testosterone (118). Shutt (119) has reported a method to assay 17 β-estradiol using a soluble macromolecule from uteri of ovarectomized ewes.

F.   Comparison of Methods of Corticosteroid Analysis.

When a comparison of the methods available for the determination of corticosteroids is made (Table VI), it is apparent that the double isotope derivative dilution method offers great advantages in sensitivity and precision. Competitive protein binding radioassays and fluorometric methods have sensitivities in the nanogram range, while spectrophotometric methods have sensitivities in the microgram range. This advantage of sensitivity and precision of the double isotope methods is offset by the very time consuming nature of this type of analysis; only a few samples can be processed each week. From the point of view of speed, simplicity

TABLE V.

Specificity of Competitive Protein Binding Radioassays.

Reference	Substances Detected[a]	Substances Not Detected
(101)	Cortisol[b] Corticosterone[b] 11-Deoxycortisol[b]	Aldosterone, Cholesterol, Tetrahydrocortisol, Tetrahydrocortisone
	Cortisone[c] Progesterone[cd]	Adrenalin, γ-Amino butyric acid, Bilirubin, Chlorpromazine, Dextrose, Digitoxin, Fructose, Histamine, Reserpine Clotted, heparinized, oxalated and hemolysed blood samples give same results
(108)	Cortisol (1) 11-Deoxycortisol (.98) Prednisolone (.92) Corticosterone (.91) Deoxycorticosterone (.58) Progesterone (.32)[d] Cortisone (.12) Prednisone (.08)	Betamethasone, Dexamethasone, Tetrahydrocortisol, Tetrahydrocortisone, Triamcinolone, Paramethasone acetate, Pregnanediol, Pregnanetriol, Methylprednisolone
(106)	Cortisol (1-1)[e] 17-Hydroxyprogesterone (.43-.47) 11-Deoxycortisol (.26-.89) Corticosterone (.1-.61) Cortisone (.07-.09) Progesterone (0-.2) Testosterone (0-.04) Desipramine HCl[f] Methotrimeprazine[f]	Betamethasone, 9α-Fluorocortisol, 9α-Fluoro-16α-hydroxycortisol, Methylprednisolone, Δ^1-17α-Methyltestosterone, Prednisone, Stilboestrol Androstenedione, Androsterone, Dehydroepiandrosterone, Etiocholanolone Estradiol, Estriol, Estrone Pregnanediol Isuprel, Thyroxine, Triiodothyronine Amitriptyline, Chlordiazepoxide, Chlopromazine, Diazepam, Imipramine, Meprobamate, Perphenazine, Prochlorperazine, Thioridazine, Trifluoperazine Phytonadione, Thiamine, Atropine, Bethanechol, Cholesterol, Chlorpheniramine, Colchicine, Digoxin, Dimenhydrinate, Diphenylhydantoin, Glucose, Mecamylamine, Oxtryphylline, Phenidione, Potassium Chloride,

Quinine, Sodium salicylate, Sucrose, Valethamate

(109)	Cortisol (1)	Progesterone
	11-Deoxycortisol (.45)^g	
	Cortisone (.32)	
	Corticosterone (.13)	

(110)^h	11-Deoxycortisol (2.1)	Aldosterone
	Testosterone (1.6)	Cortisol Acetate
	17-Hydroxyprogesterone	Deoxycorticosterone
	(1.26)	Tetrahydrocortisol
	Progesterone (1.15)	Prednisone
	Cortisol (1)	
	Prednisolone (.98)	
	Corticosterone (.57)	
	Cortisone (.20)	
	Pregnenolone (.18)	
	Androstenediol (.18)	
	17-Hydroxypregnenolone	
	(.15)	
	Androstenedione (.11)	
	Estradiol (.10)	

[a] Figures in brackets indicate response produced by equal weight of steroid relative to cortisol = 1.

[b] All detected to approximately equal extent.

[c] Detected to a lesser extent than cortisol.

[d] Effect removed by petroleum ether wash of plasma.

[e] First figure response in a Fuller's earth, ^{3}H-F system; second value for a Florisil, ^{3}H-B system.

[f] Effect removed by methylene chloride extraction.

[g] Effect removed by extraction with carbon tetrachloride.

[h] Dextran coated charcoal method.

and practicability for routinue use the spectrophotometric, fluorometric and CPB methods are much more satisfactory. The sample size used can be important where repeated determinations are necessary. For this reason the CPB method offers the greater advantage since less than 1 ml of plasma can be used. Fluorometric methods may be used for these small volumes of plasma if special microcuvettes are employed. With regard to specificity the double isotope analysis is probably the best since many purification steps are possible; however since the activity used is fairly high, radio-active contamination is possible. This would give erroneously high re-sults. Spectrophotometric and fluorometric methods are both susceptible to non-steroid interference which detracts from the specificity of these methods. The CPB method has been shown to be free of non-steroid inter-ference and has been claimed to exhibit specificity for particular ster-oids. The CPB method probably represents the best available compromise of sensitivity, precision, speed, simplicity and specificity.

TABLE VI.

Comparison of Methods for Determination of Plasma Corticosteroids.

Method	Steroid	Sensitivity or Range ^{a}	Size of Sample	Precision (s in μg/ 100 ml)	Normal Values (μg/100 ml)
UV absorption	Δ^4-3-ketosteroids (requires previous separation from impurities)		40 ml (F)		
UV absorption in H_2SO_4	Corticoids (requires previous separation; interference arises due to charring of impurities)	5-50 μg/ml	50-500 ml (F)		
Blue Tetrazolium	α-ketol steroids (some specificity due to difference in time of color development)	0.1 μg	10 ml		33.2
Zimmerman Reaction	Ketosteroids (specificity arises due to position of ketone group)				
Porter Silber	17-OHCS (subject to interference from non-steroid ketones)	0.5 μg	5-20 ml 0.5-2.4 ml (microcuvettes)		8-41.2
2,4-Dinitro-phenylhydrazine	Δ^4-3-ketosteroids (some interference from 20-ketosteroids; subject to interference from non-steroid ketones)	0-20 μg/0.5 ml	10 ml		

Method	Measures / Comments	Sensitivity	Sample volume		Normal values[a]
INH	Δ^4-3-ketosteroids (any other α, β -unsaturated ketone)	0.5-8 µg/0.5 ml	20 ml		13.3 ± 1.05 se free
Fluorescence in H_2SO_4/EtOH	11-OHCS (some specificity due to difference in time of development of fluorescence and difference in acid strength)	0.008 µg (B) 0.024 µg (F)	0.1-10 ml (depending on size of cuvette)		0.034-2.1 (F) 0.16-2.2 (B) — 13-22 (11-OHCS) 6-18 (F) 0.2-5 (B)
Glc	Separates steroids but instability of steroids and derivatives causes difficulty in quantitation	5 mµg (ecd) 25 mµg (fid) 10 mµg (argon ionization)	5 ml		
Double Isotope Derivative	F, B, E, Aldo (Separates steroids by chromatography)	0.1 µg	2-10 ml		10 (F) 0.5-2.5 (B) 1.8 (E) 1.3 (S)
Double Isotope Derivative Dilution		0.25 mµg	10-50 ml	0.35 mµg	10-11 (F) 0.3-0.7 (B) 6-10 mµg/100 ml (Aldo)
Competitive Protein Binding	F, B, S (some specificity depending on species of CBG)	1 mµg	0.01-1 ml		0.8-4.9 — 8-24 (CS) 6-18 (F)

a. of standard curve.

EXPERIMENTAL

A.   Materials and General Methods.

    1.   Chemicals.

        Steroids: Cholesterol - British Drug House, Toronto, Ontario;

                recrystallized from aqueous alcohol.

              Corticosterone - Nutritional Biochemicals Corp.,

                Cleveland, Ohio.

              Cortisol - British Drug House, Toronto, Ontario;

                recrystallized from aqueous methanol.

        Solvents: Reagent grade solvents were used without further purification except that ethanol for fluorometry was distilled.

        Others: Girard P reagent - J. T. Baker Chemical Co., Phillipsburg, New Jersey.

              Florisil 80-100 mesh - Floridin Co., Tallahassee, Florida.

              Fuller's earth technical - Fisher Scientific Ltd., Pittsburg, Pennsylvania.

              2,5-Diphenyloxazol, scintillation grade- Kent Chemicals Ltd., Vancouver, British Columbia.

              1,4-Di-[2(5-phenyloxazolyl)] -benzene, scintillation grade- Kent Chemicals Ltd., Vancouver, British Columbia.

        All other chemicals were reagent grade or better.

    2.   Radioisotopes.

        Cholesterol-4-^{14}C (30.2 mCi/mM in benzene)

        Corticosterone-1,2-T (1 Ci/mM in ethanol)

        Corticosterone-1,2-T (39.1 Ci/mM in ethanol)

        Cortisol-1,2-T (2 Ci/mM in 50% ethanol-benzene)

Cortisol-1,2-T (32 Ci/mM in 50% ethanol-benzene)

Cholesterol-T

All of the isotopes were from Amersham-Searle, Des Plaines, Illinois. The radiocompound purity of the isotopes was checked by thin layer chromatography using the following three solvent systems: chloroform:ethanol (9:1), chloroform:ethanol (7:3), and chloroform:acetone (2:8). Autoradiography of the chromatograms revealed only one spot for each of the radioactive steroids. The radioactive steroids were stored in a freezer (-15°C) prior to dilution.

3. Blood Samples.

Blood samples were collected in Vacutainers[a] containing ethylenediaminetetraacetic acid as anticoagulant. The samples were centrifuged at once at 2,000 rpm for 30 minutes. The plasma was then collected and frozen (-15°C) till used. Pooled human serum was obtained from the University of Alberta Hospital, Edmonton.

4. Liquid Scintillation Counting.

Liquid scintillation counting was done with Nuclear Chicago[b] series 720, Picker Nuclear[c] Liquimat 110 equipped with Cesium-137 external standard, or Picker Nuclear Liquimat 220 equipped with Cesium-137 external standard liquid scintillation counter. Quench correction was by the channels ratio method for the Nuclear Chicago counter, the total counts of the external standard for the Liquimat 110 and the channel ratio of the external standard for the Liquimat 220. A Digital[d] PDP-8/L computer was used in conjunction with the Liquimat 220 for computing quench correction.

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[a] Becton, Dickinson & Co., Clarkson, Ontario.
[b] Nuclear Chicago, Des Plaines, Illinois.
[c] Picker Nuclear, White Plains, New York.
[d] Digital Equipment Corporation, Maynard, Massachusetts.

For dual labelled experiments total counts of the external standard were used to calculate the absolute activity of the samples. The Liquimat 110 was used with Channel A set from 20-323 to include the tritium spectrum and the lower portion of the Carbon-14 spectrum which overlaps the tritium spectrum. Channel B was set from 450-650 to detect Carbon-14 but not tritium. Channel C was set from 600-850 to detect the external standard counts. Some Carbon-14 was also detected in Channel C but its contribution was automatically subtracted by the counter. Two sets of standards were counted: one Carbon-14, the other tritium. From these standards three graphs were plotted: tritium efficiency in Channel A, Carbon-14 efficiency in Channel B and Carbon-14 efficiency in Channel A (i.e. crossover) all versus external standard counts. Knowing the external standard counts for the unknown, the counting efficiency in each channel was read from the graph and used in the following formulae.

$$\text{DPM } ^{14}\text{C} = \frac{\text{CPM Channel B}}{\text{Eff } ^{14}\text{C}_{\text{Channel B}}}$$

$$\text{DPM } ^{3}\text{H} = \frac{\text{CPM Channel A} - (\text{DPM } ^{14}\text{C} \times \text{Eff } ^{14}\text{C Channel A})}{\text{Eff } ^{3}\text{H Channel A}}$$

When external standard counts were used for quench correction it was found that there was volume dependence. For this reason all standards and samples contained 15 ml of fluor.

The following scintillation solutions were used:

Toluene Fluor.		Bray's Solution	
PPO	4 g	Naphthalene	60 g
POPOP	0.05 g	PPO	4 g
Toluene	1 litre	POPOP	0.2 g
		Methanol	100 ml
		Ethylene Glycol	20 ml
		Dioxane	to 1 litre

Fluor solutions were stored in amber bottles in a refrigerator. Bray's solution was used where indicated and when water was present in the sample. Sample solvents were usually evaporated prior to the addition of fluor especially when the solvents were chlorinated hydrocarbons, which are very strong quenchers.

Three series of quench correction standards were used throughout the study. They were toluene-^{3}H in toluene fluor, toluene-^{14}C in toluene fluor and toluene-^{3}H in Bray's solution. The appropriate standards were used depending on the isotope and fluor used for the samples.

5. Fluorometry.

An Aminco Bowman Spectrophotofluorometer was used for the determination of fluorescence. Matched glass cuvettes (1 cm x 1 cm x 4.5 cm internal dimensions) were used. The cuvettes were cleaned by rinsing with distilled water, soaking in nitric acid overnight, and thorough rinsing with distilled water taking care to leave no fingerprints on the cuvettes. Other glassware for fluorometry was cleaned by washing with Calgonite soap. Chromic acid cleaning was not employed here. The slit arrangement used for the fluorometer was 3/16 inch slit at the photomultiplier, 1/16 inch slits at positions 2 and 5 and no slits at positions 1, 3, 4 and 6.

6. Statistical Analysis.

Standard deviation (s), standard error (se) and coefficient of variability (cv) were calculated using the following formulae:

$$ s = \sqrt{\frac{\Sigma x^2 - (\Sigma x)^2/n}{n-1}} = \sqrt{\frac{\Sigma(x - \bar{x})^2}{n-1}} $$

$$ se = \frac{s}{\sqrt{n}} $$

$$ cv = \frac{s}{\bar{x}} \times 100 $$

where   n   =   the number of X values.

Student's 't' test was used to judge the significance of dif-ferences for unpaired data. The following formulae were employed in com-puting 't':

$$t = \frac{\overline{X}_d}{s_{\overline{d}}}$$

where $\overline{X}_d$ =   mean difference = $\overline{X}_1 - \overline{X}_2$

$$s_{\overline{d}} = \sqrt{s^2\left(\frac{n_1 + n_2}{n_1 n_2}\right)}$$

$$s^2 = \frac{(n_1 - 1)\, s_1^2 + (n_2 - 1)\, s_2^2}{(n_1 - 1) + (n_2 - 1)}$$

$$s_1^2,\ s_2^2 = \text{sample variance} = \frac{\Sigma X^2 - (\Sigma X)^2/n}{n-1}$$

$\overline{X}_1,\ \overline{X}_2$ =   sample means

$n_1,\ n_2$ =   sample size

The degrees of freedom for 't' is $n_1 + n_2 - 2$.

The constants for the regression equation $Y = a + bX$ were cal-culated using the following formulae:

$$b = \frac{\Sigma XY - \dfrac{\Sigma X\, \Sigma Y}{n}}{\Sigma X^2 - \dfrac{(\Sigma X)^2}{n}}$$

$$a = \overline{Y} - b\overline{X}$$

where b =   slope

a =   intercept

X =   independent variable

Y =   dependent variable

n =   number of observations

The standard deviation (s_b) of the slope was determined using the formulae (120):

$$s_b \ = \ \sqrt{V_b}$$

where V_b = variance of slope

$$= \ \frac{V_y}{\sum(X - \bar{X})^2}$$

$$V_y \ = \ \frac{\sum Y^2 - n\bar{Y}^2 - b^2 (\sum X^2 - n\bar{X}^2)}{n-2}$$

Other variables defined as above.

The standard deviation of X, estimated using this regression equation, could be predicted for m determinations of X using the formulae (120):

$$s_x \ = \ \sqrt{V_x}$$

where $V_x \ = \ \dfrac{V_y}{b^2} \left[\dfrac{1}{m} + \dfrac{1}{n} + \dfrac{(Y - \bar{Y})^2}{b^2 \{ \sum (X - \bar{X})^2 \}} \right]$

m = number of determinations of X

n = number of points used to determine the regression line

Other variables defined as above.

Analysis of variance and Duncan's multiple range test were done by the method given in Steel and Torrie (121, 122).

Computation of statistics was done using a Digital PDP-8/L computer.

B.   Application and Results.

1.   Fluorometric Assay of Cortisol and Corticosterone.

a.   Standard curves.

(1) Preparation of fluorescence reagent.

Sulfuric acid (reagent grade) was added slowly with constant stirring to 95% ethanol which was kept cool in an ice bath. The rate of addition of acid was sufficiently slow to prevent the temperature of the mixture from rising above room temperature. In order to prevent the entry of atmospheric dust into the reagent a narrow necked flask was used for preparation and storage. Dust in the reagent is reported to increase the blank fluorescence (37). Because of poor stability of the reagent it was prepared just prior to use. Sufficient sulfuric acid was used to prepare reagent of final concentration of 60 and 75%.

(2) Preparation of standard curve.

Cortisol and corticosterone standards in the desired range were prepared from stock standard solutions of the steroids. The solvent was evaporated in vacuo at 25°C. To each standard was added 1.5 ml of the desired sulfuric acid-ethanol reagent. The standards were mixed, covered and incubated at 45°C for 20 minutes. After cooling in an ice bath the fluorescence intensity was determined at activation wavelength 470 mμ and emission wavelength 530 mμ. All readings were taken within 30 minutes of the completion of the incubation and cooling.

A standard curve was prepared by plotting the number of micrograms of steroid per millilitre of reagent versus the percent relative fluorescence. The plot was described by a linear regression equation determined by the method of least squares.

All standard curves were linear over the concentration range 0.005 to 1 μg/ml. For corticosterone, when 60% sulfuric acid-ethanol reagent was

used the resulting graph could be described by the equation $Y = 58.22 X + 0.42$, where Y = % relative fluorescence and X = µg of steroid/ml of reagent. The slope factor had a standard deviation of 1.22. If duplicate estimates of a 0.1 µg/ml sample were made, the standard deviation of X would be 0.0335.

Cortisol did not fluoresce as strongly as corticosterone when treated with 60% sulfuric acid-ethanol reagent. The equation for the graph was $Y = 11.02 X + 1.52$, where the standard deviation of the slope was 0.682. Duplicate estimates of a 0.1 µg/ml sample using this standard curve would give an X value with a standard deviation of 0.0889.

The fluorescence of corticosterone was reduced when 75% sulfuric acid-ethanol was used as the reagent in preparation of the standard curve. The equation describing the line was $Y = 36.44 X + 0.23$. The standard deviation of the slope was 3.78, while the standard deviation of X where X is 0.1 µg/ml and duplicate determinations are made would be 0.166. It can be seen that variability of the results was increased with the 75% reagent compared to those results observed with the 60% reagent. The variation of the standard curve is unacceptable since the standard deviation of X would be greater than X itself.

The fluorescence of cortisol in 75% sulfuric acid-ethanol reagent was increased relative to the cortisol fluorescence in 60% reagent. The equation for the standard curve was $Y = 23.99 X + 1.69$ with a slope standard deviation of 2.17. The standard deviation of X where X is 0.1 µg/ml and duplicate estimates are made, would be 0.130, which is not acceptable since again the standard deviation of X exceeds the value of X.

Figure 7 demonstrates the various standard curves that were determined. Each point on the graph is the average of three or more standards prepared at that concentration. Figure 8 shows the individual

Figure 7. Fluorescence Standard Curves

41.
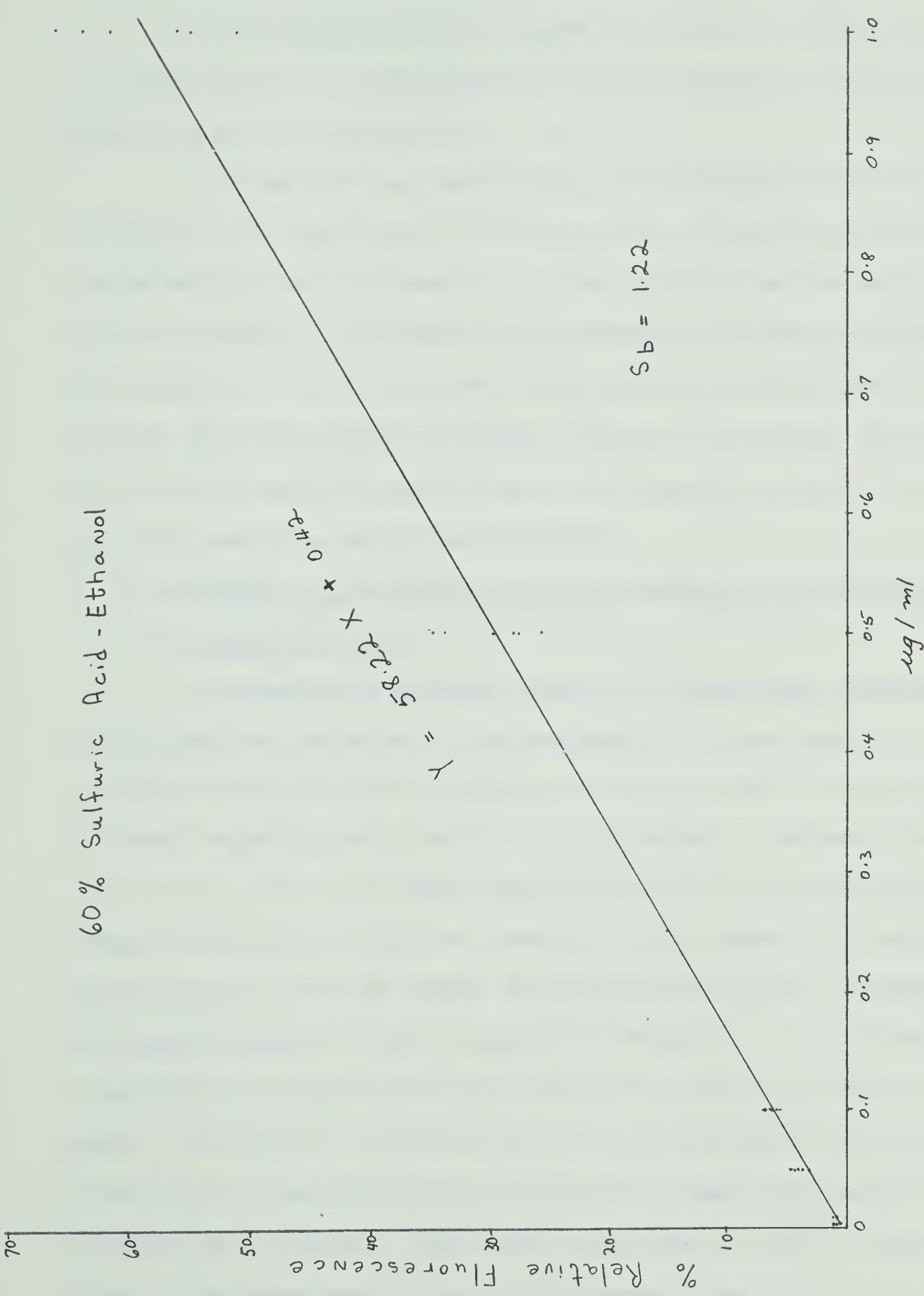
Figure 8. Corticosterone Fluorescence Standard Curve

values for the determination of the corticosterone standard curve using 60% sulfuric acid-ethanol reagent.

It was concluded that for assay of samples containing only one of either cortisol or corticosterone, that 60% sulfuric acid-ethanol reagent gave more reliable results.

Incubation for a longer period of time (45 minutes) caused a decrease in the fluorescence of corticosterone at both 60% and 75% acid concentrations, and a decrease in the fluorescence of cortisol at 75% acid concentration. An increase in fluorescence of cortisol at 60% acid was observed. An increase in the variability of fluorescence was also observed for both strengths of reagent and for both steroids. Because the effect of longer incubation was not consistent, it was felt that the 20 minute incubation period was preferable.

b. Resolution of cortisol and corticosterone in a mixed sample.

(1) Matsumara method.

Matsumara (39) described a method for individually determining cortisol and corticosterone in the same sample which was based on the difference in the relative fluorescence of cortisol and corticosterone at different concentrations of sulfuric acid in ethanol. The sample was split in two, and one half was treated with 75% sulfuric acid-ethanol reagent while the other half was treated with 60% reagent. The relative fluorescence of these two samples was determined and used to determine the apparent amount of total corticoids by reference to corticosterone standard curves prepared with both 75 and 60% sulfuric acid-ethanol reagent. The ratio of fluorescence of cortisol/corticosterone was determined in both 75 and 60% sulfuric acid-ethanol reagent with samples containing 1 μg of steroid. These ratios were termed 'a' and 'b' respectively. They were used in a set of simultaneous equations in order to

determine the amount of each steroid in the sample. The equations were:

$$X + a\,Y = c \quad (1)$$

$$X + b\,Y = d \quad (2)$$

where X, Y = corticosterone and cortisol concentrations, respectively in the unknown sample

c, d = apparent corticoid concentrations in 75% and 60% acid, respectively

a, b = ratio fluorescence F/B in 75% and 60% acid, respectively.

In order for this method to be valid it is necessary that the ratios 'a' and 'b' be constant at all concentrations of steroid. To determine if this was the case the ratio of fluorescence of F/B was determined for concentrations of each steroid from 0.01 to 1 µg/ml. The fluorescence was developed and determined as described for the standards. The results are shown in Table VII. Analysis of variance showed that the ratios were not constant at all concentrations of steroid tested.

TABLE VII.

Fluorescence Ratio[a] of Cortisol/Corticosterone in Sulfuric Acid-Ethanol Reagent.

| | Concentration of Steroid (µg/ml) | | | | |
	0.01	0.05	0.10	0.50	1.00
Ratio 'a'	1.59	1.22	1.16	0.53	1.09
	0.82	1.39	1.12	0.46	1.41
	1.32	1.64	1.24	0.52	0.58
					0.57
					0.71
					0.57
Average	1.24[ef]	1.42[f]	1.17[ef]	0.50[d]	0.82[de]
Ratio 'b'	0.44	0.48	0.54	0.20	0.21
	0.56	0.44	0.81	0.26	0.23
	0.47	0.55	0.98	0.27	0.20
					0.23
					0.22
					0.22
Average	0.49[c]	0.49[c]	0.78	0.24[b]	0.22[b]

[a] Each value is a ratio of fluorescence F/B for equal weights of each steroid. Ratio 'a' is for 75% sulfuric acid-ethanol; ratio 'b' is for 60% sulfuric acid-ethanol.

[b-f] Mean values not followed by the same letter are significantly different at the 5% level by Duncan's multiple range test.

Several mixed samples containing both cortisol and corticosterone in various concentrations were assayed by fluorometry. These samples were treated in manner similar to that described for the standards. Application of the calculation method for resolving cortisol and corticosterone was attempted. Since the ratios 'a' and 'b' were found to vary with concentration of steroid, it was necessary to arbitrarily chose a set of ratios at one concentration in order to do the calculations. When the simultaneous equations were solved for these samples, large

errors in the estimate of steroid concentration resulted. The recovery in mixed samples containing 0.01 to 1 μg/ml of each steroid ranged from 13 to 570% of the correct result, with many negative values also observed. Table VIII shows the results obtained by the calculation method of re-solving the steroids.

TABLE VIII.

Determination of Cortisol and Corticosterone in Mixed Samples of Known Concentration.

Known conc[a]		% Rel Fluor		Apparent conc[b]		Calc conc[ac]		% Recovery	
F	B	75%	60%	75%	60%	F	B	F	B
1.0	0.5	51.0	28.1	1.4	0.42	1.6	0.066	160	13
"	"	53.0	22.0	1.5	0.37	1.9	-0.038	190	-ve
"	"	52.5	14.8	1.4	0.25	1.9	-0.17	190	-ve
1.0	0.1	25.9	13.0	0.44	0.22	0.36	0.14	36	140
"	"	25.6	7.72	0.70	0.13	0.94	-0.076	94	-ve
"	"	23.5	7.87	0.64	0.13	0.84	-0.054	84	-ve
0.1	0.1	16.2	6.04	0.44	0.097	0.57	-0.027	570	-ve
"	"	7.94	11.2	0.21	0.18	0.050	0.17	50	169
"	"	6.14	6.02	0.16	0.096	0.11	0.073	110	73
0.1	0.05	5.64	3.67	0.15	0.056	0.16	0.022	160	44
"	"	5.34	4.12	0.14	0.064	0.13	0.037	130	74
"	"	5.14	3.07	0.13	0.046	0.14	0.016	140	32
0.1	0.01	2.89	2.31	0.073	0.033	0.066	0.019	66	190
"	"	3.99	2.35	0.10	0.033	0.11	0.0088	110	88

[a] μg of steroid/ml of reagent.

[b] Calculated from % relative fluorescence using the following equations:

$Y = 36.44 X + 0.23$ (for 75% reagent)
$Y = 58.22 X + 0.42$ (for 60% reagent)
where Y = % relative fluorescence
   X = apparent concentration in terms of B.

[c] Calculated using the simultaneous equations
where 'a' = 0.822, and 'b' = 0.218.
These ratios were determined at steroid concentration of 1 μg/ml.

(2) <u>Criteria for separation of cortisol and corticosterone.</u>

In view of the poor results obtained by the calculation method of resolving cortisol and corticosterone, it was necessary to find another means to estimate them individually. Since the 60% sulfuric acid-ethanol reagent gave a more reliable standard curve with both cortisol and corticosterone it could be used for assay of the separated steroids. The degree of separation of the steroids necessary will depend on the relative fluorescence of the steroids and on the amount of interference permitted. If 5% interference is chosen as the maximum allowable level, the ratio of concentration of one steroid to the other required in the final sample used in fluorescence assay, will be the product of 5/100 and the ratio of fluorescence of the desired steroid to that of the interfering steroid. From Table VII the ratio of fluorescence of cortisol to corticosterone in 60% sulfuric acid-ethanol varies from 0.22 to 0.78. For the determination of corticosterone the interference of cortisol will be most pronounced where the ratio is highest; therefore, the ratio 0.78 was used to calculate the maximum permissible cortisol to corticosterone ratio in the final sample. The maximum F/B concentration in the assay sample would be (5/100) x (1/0.78) = 0.064. In normal plasma the concentration of cortisol relative to corticosterone is about 10/1, thus for fluorometric assay of corticosterone the concentration ratio of F/B must be reduced from 10/1 to 0.064/1. In order to achieve this one must get complete recovery of corticosterone and only $\frac{0.064}{10}$ x 100 = 0.64% recovery of cortisol in the corticosterone phase used in the assay.

Since cholesterol is present in plasma in such a large concentration (200 mg/100ml), it constitutes an interfering steroid despite its low relative fluorescence (0.005 compared to corticosterone = 1) (39).

Table IX shows the required relative concentration of an inter-

fering steroid such that not more than 5% of the fluorescence is derived from that steroid. Bearing in mind the normal concentration of the desired steroid and the interfering steroid, the percentage allowable crossover has been calculated. Since two steroids were considered, if the criteria in Table IX are just met the interference will be 10%. Of course, other steroids may interfere and raise the interfering fluorescence above this level.

TABLE IX.

Criteria for Judging Suitability of Separations for Individual Fluoro-
metric Determination of Cortisol and Corticosterone.

Steroid	Relative[a] Fluorescence	Required Relative Concentration in Final assay sample	Normal[b] Relative Concentration	% Cross-over[c] Allow-able
For Determination of B				
F/B	0.78/1	0.064/1	10/1	0.64
Chol/B	0.005/1	10/1	$2 \times 10^5/1$	0.005
For Determination of F				
B/F	4.5/1	0.011/1	0.1/1	9.1
Chol/F	0.019/1	2.65/1	$2 \times 10^4/1$	0.014

[a] Values for B and F from Table VII, those for cholesterol from Matsumara (39). All values apply to 60% sulfuric acid-ethanol.

[b] Based on normal concentration of cortisol 10 μg/100 ml plasma, corticosterone 1 μg/100 ml and cholesterol 200 mg/100 ml.

[c] Assuming 100% recovery of desired steroid and 5% interference from the undesired steroid in the fluorescence produced.

Separation of the steroids was attempted by solvent partitioning. In order to attain uniformity all extraction and washing was done by shaking one minute on the Vortex Junior Mixer. Low specific activity ^{3}H-steroids were used in these experiments.

(a) <u>Carbon tetrachloride/water partitioning of corticosterone and cortisol.</u>

Known amounts of ^{3}H-corticosterone and ^{3}H-cortisol were dissolved in 2 ml of water in a stoppered centrifuge tube. This solution was extracted with 20 ml of carbon tetrachloride. The phases were separated by centrifugation and the water phase was backwashed with 20 ml of carbon tetrachloride which was subsequently discarded. The initial carbon tetrachloride was backwashed with 2 ml of water. The cortisol being more polar will favour the water phase, while the corticosterone will go into the carbon tetrachloride. Table X shows the results of the separation as determined on two different days.

TABLE X.

Separation[a] of Corticosterone and Cortisol Using Carbon Tetrachloride/
Water Partitioning.

Number of Samples	Water Phase		Carbon Tetrachloride Phase	
	F Recovery	B Crossover	B Recovery	F Crossover
7	58.2 ± 2.8			3.9 ± 0.5
9		4.9 ± 1.0	65.0 ± 3.2	

[a] Mean % ± standard deviation.

Since the separation of cortisol and corticosterone by this method seemed feasible, further experiments were done using this method in combination with other steps to separate cholesterol from the two corticoids.

(b) <u>Separation of cholesterol from cortisol and corticosterone.</u>

Attempts to use the Girard P reagent method described by Matsumara (39) were unsuccessful, therefore solvent partitioning was tried in order to separate cholesterol. Two solvent partitioning methods were

used in conjunction with the carbon tetrachloride/water partitioning procedure in an attempt to individually recover cortisol and corticosterone from serum without recovering cholesterol with them. In each method the sample consisted of 4 ml of human serum to which known amounts of ^{3}H-cortisol or ^{3}H-corticosterone had been added. The samples in stoppered centrifuge tubes were mixed thoroughly before extraction.

Procedure 1. The serum sample was washed with 5 ml of petroleum ether, then extracted two times with 8 ml of chloroform. The chloroform was washed with 1 ml of 0.1 N sodium hydroxide then evaporated and the residue was dissolved in 2 ml of water. This was partitioned against carbon tetrachloride as described previously.

Procedure 2. The serum sample was extracted two times with 8 ml of chloroform then the extract was washed with 0.1 N sodium hydroxide. The extract was evaporated and the residue was dissolved in water (2 ml). This solution was then washed with petroleum ether (5 ml) to remove cholesterol. The water was then partitioned against carbon tetrachloride as described in the previous section. Table XI shows the results of these procedures.

TABLE XI.

Separation of Cortisol, Corticosterone and Cholesterol.

Method	Phase	Steroid[a]		
		Corticosterone	Cortisol	Cholesterol
1.	Pet ether	$(-^b)$	$(-^b)$	$-^b$
	Water	(4.4 ± 0.5)	4.1 ± 0.5	(0.4 ± 0.5)
	CCl$_4$	39.0 ± 3.1	(19.6 ± 2.9)	(17.3 ± 13.0)
2.	Pet ether	(4.1 ± 1.1)	(1.7 ± 0.2)	9.1 ± 3.8
	Water	(10.0 ± 3.5)	14.9 ± 4.1	(20.7 ± 6.9)
	CCl$_4$	14.7 ± 15.3	(6.3 ± 1.6)	(7.2 ± 2.6)

[a] Mean % ± standard deviation. Each value average of two or more determinations. Those values in parentheses are crossover; other values are recovery.

[b] Petroleum ether gel formed on extraction so fraction not counted.

Reference to Tables IX and X shows that there was adequate separation of corticosterone and cortisol for the fluorometric assay of cortisol even when correction for failure to get 100% recovery of cortisol was made. On the other hand the isolation of corticosterone was not satisfactory because of the excessive crossover from cortisol. Removal of cholesterol by partitioning was also not satisfactory (Table XI); furthermore the results indicate that the partitioning technique is unsatisfactory when applied to serum samples.

Although the partitioning method for separation has the advantage of simplicity, relative speed and ability to process a large number of samples, it does not adequately remove cholesterol from the sample. The presence of this cholesterol in the assay sample would likely elevate the fluorometric estimates of cortisol and corticosterone. Chromatographic means might be employed in order to separate cholesterol from the corticoids and to separate cortisol and corticosterone. This would add additional problems of increased background fluorescence arising from the chromatographic support medium. Chromatography would complicate the procedure and reduce recovery since quantitative elution is difficult to achieve. Thus, the spectrofluorometric method is limited to samples containing either cortisol or corticosterone alone. Cholesterol must be removed by methods other than solvent partitioning in order to make this assay technique suitable for the determination of the former two steroids in plasma samples.

2. <u>Competitive Protein Binding Radioassay of Cortisol and Corti-costerone.</u>

Because several attempts to use the protein binding method as reported by Murphy (106) were not successful, a study of conditions necessary for assay using this technique was initiated. The approach was to first determine adequate conditions for use of the assay on samples of steroid which were of known concentration and which were known to be of pure composition. The method finally developed can thus be applied for the estimation of the steroids in plasma samples.

a. <u>Optimum conditions for use of adsorbent for separation of free steroid from protein-bound steroid.</u>

(1) <u>Adsorption of free steroids.</u>

For the determination of adsorption of free steroids, a measured quantity of Fuller's earth or Florisil was added to 1 ml of a solution of cortisol-1,2-T or corticosterone-1,2-T which had been cooled to at least 10°C for 15 minutes. The tubes were shaken for 2 minutes on a horizontal mechanical agitator, cooled for a further 10 minutes in an ice bath, and then centrifuged at 4000 rpm for 2 minutes. The supernatant, containing the unadsorbed corticoid, was removed with a pasteur pipette. One half ml of this solution was added to 10 ml of Bray's solution and the sample was counted. Knowing the amount of activity initially present per half ml of solution, it was possible to calculate the percentage of adsorbed steroid.

Except where indicated otherwise, adsorbents were measured in small plexiglass spoons which were calibrated for each adsorbent. The spoons (A and B) were made by drilling holes of diameter 0.2 and 0.6 cm in a rectangular piece of plexiglass (1 x 12.5 x 0.6 cm). The spoons were loosely filled with adsorbent and the contents levelled with the

surface of the spoon. The capacity of these spoons for the two adsorbents is shown in Table XII.

TABLE XII.

Calibration of Spoons.

Adsorbent	Capacity ± standard deviation (mg)	
	A	B
Fuller's earth	10.32 ± 1.02	59.14 ± 1.58
Florisil	8.38 ± 0.81	41.80 ± 0.51

(a) Effect of organic solvents on adsorptive power of Fuller's earth.

Preliminary experiments to determine the general conditions for adsorption were conducted using various quantities of Fuller's earth and ^{3}H-cortisol. Use of an organic solvent (ethanol:benzene::1:1) as a medium for adsorption studies indicated that adsorption of cortisol by Fuller's earth was severely decreased. If the solvent was aqueous ethanol (0.004 or 2%) substantial adsorption of cortisol was observed.

(b) Effect of acid washing of Fuller's earth on adsorption of ^{3}H-cortisol.

In an effort to increase the adsorptive powers of Fuller's earth, it was acid washed. It is known that the bleaching power of Fuller's earth, which is attributed to its adsorptive power, is enhanced by treatment of the Fuller's earth with dilute acid (123). A batch of Fuller's earth was washed with 1 N hydrochloric acid and dried 16 hours at 100°C. It was then powdered to 60 mesh size of particles and stored in a vacuum desiccator. The adsorption of cortisol by the techical grade and the acid washed Fuller's earth was then compared (Table XIII).

TABLE XIII.

Adsorption of ^{3}H-Cortisol[a] by Acid Washed and Technical Fuller's earth.

Weight of Fuller's earth (mg)	% Adsorbed[b]	
	Technical	Acid Washed
10	36.1	22.4
20	53.9	57.5
40	70.3	79.5

[a] ^{3}H-Cortisol was dissolved in 2% ethanol/water; cortisol concentration was 0.28 mμg/ml.

[b] Average of duplicates.

An analysis of variance (random factorial design) showed that there was no detectable significant difference ($F < 1$) in adsorption for these two batches of Fuller's earth. It did not matter, therefore which type of Fuller's earth was used.

(c) Adsorption of free steroid by various weights of adsorbent.

In order to determine the quantity of adsorbent required for maximum adsorption of unbound steroid, the percent adsorption of steroid was determined for a number of different weights of adsorbent. Tables XIV and XV demonstrate these relationships.

TABLE XIV.

Adsorption of ^{3}H-Cortisol[a] by Various Weights of Fuller's earth.

Weight of Fuller's earth (mg)	% Adsorbed[b]
10	33.8
20	56.7
40	77.6[ce]
70	83.8[cde]
90	88.4[cd]
120	87.4[cd]

[a] ^{3}H-Cortisol dissolved in 0.004% ethanol/water; cortisol concentration 22 mμg/ml.

[b] Average of duplicates.

[c] Values followed by this letter not significantly different at 1% level by Duncan's multiple range test.

[d-e] Values followed by the same letter not significantly different at the 5% level by Duncan's multiple range test.

Analysis of variance (random design) showed that there was a significant difference ($P < 0.005$) in adsorption when different weights of Fuller's earth were used. Examination of the ranked means by Duncan's multiple range test at the 1% level showed that there was no detectable difference in the adsorption of cortisol for weights of Fuller's earth in the range 40 to 120 mg, while 10 and 20 mg adsorbed less cortisol than the rest, and were significantly different from each other. The adsorption of cortisol by 40 to 120 mg of Fuller's earth is 84.3 ± 5.0 (s) %. The optimum concentration of Fuller's earth should be chosen in this range so that slight errors in measuring the adsorbent will not significantly alter the adsorption of cortisol.

TABLE XV.

Adsorption of ^{3}H-Corticosterone by Various Weights of Florisil.

Weight of Florisil (mg)	% Adsorbed[a]
8	64.3
10	75.1
20	86.6[b]
40	86.1[b]
60	86.8[b]
80	92.9[b]
100	91.2[b]
120	93.1[b]

[a] Average of duplicates.

[b] Values followed by this letter not significantly different at 1 and 5% level by Duncan's multiple range test.

Analysis of variance (random design) showed there was a significant difference ($P < 0.005$) in the % adsorbed corticosterone by different weights of Florisil. Duncan's multiple range test indicated that there was no detectable difference in adsorption from 20 to 120 mg Florisil while 8 and 10 mg gave lower adsorption, the latter two also being different from each other. The adsorption of corticosterone by 20 to 120 mg of Florisil was 89.4 ± 3.5 (s) %, 75.0 ± 4.2 (s) % for 10 mg, and 64.3 ± 5.2 (s) % for 8 mg.

(d) Adsorption of various weights of steroid by adsorbent.

Since removal of free steroid from solution is not complete, it is necessary to establish that the amount adsorbed is constant at various weights of steroid. For this reason, experiments were conducted to determine if this criterion was met. Tables XVI and XVII illustrate the results obtained.

TABLE XVI.

Adsorption of Various Weights of ^{3}H-Cortisol by Fuller's earth.

Weight of Cortisol (mμg)	% Adsorbed[a] Weight of Fuller's earth (mg)	
	60	120
1	-	78.5
5	-	88.4[c]
10	-	90.1[c]
10.4	84.5[b]	-
13.5	82.0[b]	-
20	-	90.3[c]
22	81.8[b]	-
40	-	90.5[c]

[a] Average of duplicates.

[b-c] Values followed by the same letter not significantly different at 1 and 5% level.

Analysis of variance (random design) for data using 60 mg of Fuller's earth indicated that from 10.4 to 22 mμg of cortisol adsorption was constant at 82.8 ± 1.8 (s) %. Analysis of variance for data at 120 mg indicated that there was a significant difference ($P < 0.005$) in adsorption for various weights of cortisol. Application of Duncan's multiple range test to the ranked means revealed that adsorption was constant for weights of cortisol from 5 to 40 mμg but was significantly lower for 1 mμg of cortisol. The adsorption of cortisol in quantities from 5 to 40 mμg by 120 mg of Fuller's earth was 89.9 ± 1.1 (s) %.

TABLE XVII.

Adsorption of Various Weights of ^{3}H-Corticosterone by Florisil.

Weight of Corticosterone (mμg)	% Adsorbed[a] Weight of Florisil (mg)					
	8	10	16	20	90	Average
1.4	82.2	75.6	88.3	86.2	-	83.1[b]
5.4	84.2	79.3	88.0	88.1	-	84.9[b]
10.4	83.2	84.2	88.4	85.8	-	85.4[b]
17	-	-	-	-	89.0[c]	
20.4	83.2	81.2	87.0	85.0	-	84.1[b]
21	-	-	-	-	88.8[c]	
26	-	-	-	-	87.7[c]	
36	-	-	-	-	88.1[c]	
Average	83.2[d]	80.1[d]	87.9[e]	86.3[e]		

[a] Average of duplicates.

[b-e] Values followed by the same letter not significantly different at 5% level.

Analysis of variance (random factorial design) for Florisil weights of 8 to 20 mg detected no significant difference between the adsorptions for different weights of corticosterone. On the other hand, a significant difference in adsorption for different weights of Florisil was demonstrated. This latter finding confirms a previous observation (section (c)). In the present experiment 8 and 10 mg Florisil adsorbed the same amount of corticosterone (81.6 ± 3.7 (s) %) while 16 and 20 mg Florisil adsorbed more (87.1 ± 1.4 (s) %). When these results are compared to the results in Table XV the adsorption for 8 and 10 mg is higher than previously determined and the result for 16 and 20 mg is similar to the previous results.

Analysis of variance (random design) for 90 mg of Florisil showed that there was no difference in adsorption of corticosterone in the range 17 to 36 mμg, the quantity adsorbed being 88.4 ± 1.0 (s) %. This amount of adsorption is in good agreement with the results in

Table XV.

From these experiments it can be concluded that, except for the case of very low concentrations of cortisol, the percent adsorption of cortisol and corticosterone is constant for various weights of steroid at selected weights of adsorbent.

(2) Separation of bound and free steroid by adsorbents.

Since it was established that reproducible, though not quantitative, removal of free steroid from solution can be achieved by use of adsorbents, it was desired to show that significant protein binding is possible in the presence of adsorbents, and that a separation of free and bound steroid can be made by use of adsorbents. To determine the percent steroid bound to protein, paired samples of radioactive steroid (in 1 ml of 0.004% aqueous ethanol) were treated identically except that one contained the specific binding protein and the other contained no protein. The tubes were warmed to 45°C for 5 minutes to promote rapid equilibration of the steroid with the protein. The samples were then cooled to 0°C for 10 minutes such that dissociation of the steroid-protein complex would be slowed. A relatively static system of free and bound steroid would result. A measured quantity of adsorbent was added and the tubes were shaken for 2 minutes. The tubes were returned to the ice bath for 15 minutes, then centrifuged at 4000 rpm for 2 minutes. The supernatant was removed. One half ml of this supernatant was added to 10 ml of Bray's solution and this sample was counted. The tubes were kept in an ice bath after centrifugation to prevent any dissociation of the steroid-protein complex prior to the pipetting of the sample. The samples without protein contain unadsorbed steroid in the supernatant, while those which contained the specific binding protein have unadsorbed steroid plus protein-bound steroid in the supernatant. The difference in

the percent of original activity between the protein and non-protein samples represents the percent of steroid bound to protein. Tables XVIII and XIX show the relationships between the various weights of adsorbents and the amount of steroid bound by the protein.

TABLE XVIII.

Influence of Fuller's earth on Protein-Bound[a] ^{3}H-Cortisol.

Weight of Fuller's earth (mg)	% Protein Bound Cortisol[b] Weight of Cortisol (mµg)			
	10.4	13.5	22	Average
10	36.1	34.2	12.4	27.6[def]
20	45.6	41.2	25.4	37.4[cde]
40	43.0	45.0	20.6	36.2[cde]
70	37.6	42.2		39.9[ce]
90	34.0	30.9	18.5	27.8[def]
120	21.8	18.0	13.5	17.8[f]
Average	36.4[g]	35.2[g]	18.1	

[a] Source of CBG was 5% human plasma.

[b] Average of duplicates.

[c-d] Values followed by same letter not significantly different at 5% level.

[e-f] Values followed by same letter not significantly different at 1% level.

[g] Values followed by this letter not significantly different at 1 and 5% levels.

Analysis of variance (random factorial design) showed a significant difference ($P < 0.005$) in protein binding for both changes in cortisol weight and changes in adsorbent weight; however the interaction was not significant. Protein binding was significantly lower when the weight of cortisol was 22 mµg compared to 13.5 and 10.4 mµg, which were not found to be different. This effect is expected since a change in protein binding should occur with a change in steroid weight (this is the basis

of the assay). It also implies that the difference between 10.4 and 13.5 mµg cannot be detected under these conditions. Protein binding was constant in the range 10 to 90 mg of Fuller's earth (Duncan's at 1% level). For 10.4 to 13.5 mµg of cortisol the binding was 39.0 ± 6.2 (s) % (10 to 90 mg Fuller's earth) and for 22 mµg of cortisol the bound fraction was 20.4 ± 7.1 (s) %. For maximum sensitivity maximum protein binding is desirable; therefore an optimum weight of Fuller's earth would be in the range 10 to 90 mg. The weight 60 mg was chosen because it was in the range giving maximum protein binding and also in the range giving maximum adsorption of free cortisol. Furthermore a spoon measuring this quantity of Fuller's earth was already available. Since this weight is in the middle of the ranges slight errors in adsorbent weight would have little effect on the results of assay.

TABLE XIX.

Influence of Florisil on Protein-Bound[a] ^{3}H-Corticosterone[b].

Weight of Florisil (mg)	% Protein-Bound Corticosterone[c]
8	46.6
10	30.9
20	13.6[d]
40	9.0[d]
80	8.1[d]
120	5.4[d]

[a] Source of CBG was 0.5% mouse plasma.

[b] Corticosterone concentration was 0.88 mµg/ml.

[c] Average of duplicates.

[d] Values followed by this letter not significantly different at 1 and 5% levels by Duncan's multiple range test.

Analysis of variance (random design) indicated that there was a significant (P = 0.01) difference in the percent protein-bound corticosterone with different weights of Florisil. Duncan's multiple range test on ranked means indicated that the percent protein-bound steroid was constant in the range 20 to 120 mg Florisil, that 8 and 10 mg gave a higher percent protein-bound and that 8 and 10 mg were different from each other. Average protein-bound corticosterone for 20 to 120 mg Florisil was 9.0 ± 3.7 (s) %. This binding being quite low would give poor sensitivity; therefore 8 or 10 mg of adsorbent must be chosen for use in the assay despite the fact that a small error in measuring would give a marked change in the amount of protein-bound steroid. This would lead to a lowering of precision for the assay.

b. <u>Preparation of standard curves.</u>

The preparation of standard curves was done to demonstrate that the percent protein-bound steroid did vary with the weight of steroid; furthermore it was necessary to determine a useful range for the assay, and to show that reliable linear graphs of reciprocal of activity versus weight of steroid could be established. Actual determination of the % protein-bound steroid is not necessary in making the standard curve. It will be recalled that % protein-bound steroid was determined by finding the difference in % activity after treatment with adsorbent for paired samples of steroid, one containing protein and the other containing no protein. Since the % activity removed by adsorbent from the samples containing no protein (i.e. the samples containing free steroid only) is constant at all weights of steroid, it can be seen that the % protein-bound steroid is proportional to the % activity removed from the protein sample by the adsorbent. Furthermore, in a given assay the initial activity of the protein solution is the same for all stand-

ards and samples so the case can be further simplified to say that % pro-
tein-bound steroid is proportional to the activity remaining in the pro-
tein sample after treatment with adsorbent. The relationship of % protein-
bound steroid to weight of steroid is such that the reciprocal of % bound
gives a linear relationship when plotted against steroid weight.

Initial attempts at making standard curves were made using the
amounts of adsorbent determined as optimum in the previous section, and
the CBG source and concentration as suggested by the data of Murphy (106).

The procedure for preparing standard curves was as follows.
Standard quantities of steroid were measured in duplicate in the desired
range for the assay. Usually five weights in the selected range were
used; one of these weights was always zero. To each tube in the series
of standards 1 ml of CBG isotope solution was added. These were mixed
intermittently for 5 minutes while being heated in a water bath at 45°C.
During this step radioactive steroid on the CBG equilibrates with the ad-
ditional steroid in the tube, resulting in a decrease in the activity of
the CBG. Dissociation of CBG steroid complex is very rapid at 45°C so
equilibrium of the added steroid and the isotope solution is quickly
reached. The tubes were next cooled to 0°C for 15 minutes in order to
stop any further exchange of free and bound steroid. Actually exchange
may still occur but it will be very much slower. The adsorbent was then
added to the tubes and they were shaken for 2 minutes on a horizontal
mechanical agitator. The adsorbent removes the free steroid in the sol-
ution and provides a means of separating bound and free steroid. The
tubes were cooled for 10 minutes in an ice bath and centrifuged in a
refrigerated centrifuge at 4°C. No more tubes should be processed than
can be centrifuged at one time. In this laboratory, the maximum number
of tubes that were handled at one time was 24. The supernatant was

collected and in the case of Fuller's earth, recentrifuged. The super-
natants were transferred to second tubes with pasteur pipettes and 0.5 ml
was added to 10 ml of Bray's solution. Prior to pipetting the tubes were
kept in an ice bath. Keeping the samples cool at all stages after the
equilibration is necessary to prevent further exchange of radioactive
steroid which would change the activity of the protein-bound fraction.
The samples were counted by liquid scintillation at least two times. The
counting time was sufficient to give 2% counting error with 2 sigma stat-
istics. A standard curve was prepared by plotting the weight of steroid
versus the reciprocal of activity in the protein-bound fraction (the
reciprocal of activity was expressed as milliseconds per disintegration).
As the amount of steroid increases, the displacement of the radioactive
label from the CBG also increases; thus, the activity of the protein-
bound fraction decreases. This means that a plot of reciprocal of activ-
ity against weight of steroid will have a positive slope. Since the re-
lationship between weight and reciprocal of activity is linear, the curve
was fitted by the method of least squares. The resulting regression
equation was used to determine the concentration of steroid in unknown
samples.

The standards were made from stock solutions of cortisol or
corticosterone which were 10 μg/ml in redistilled ethanol. As required,
0.1 ml of the stock was diluted to 10 ml with redistilled ethanol pro-
ducing a solution of concentration 10 mμg/0.1 ml. The ethanol was evap-
orated from the standards prior to addition of the CBG isotope solution.

The CBG isotope solution was prepared by diluting the desired
quantity of plasma with distilled water, then adding the tritium labelled
steroid in a small quantity of ethanol. The solution was then brought to
volume with distilled water.

The above procedure for preparing the standard curve and processing the samples may be summarized as follows:

1. Add 1 ml of CBG isotope solution to the evaporated standard or sample; mix and heat to 45°C for 5 minutes.

2. Cool on an ice bath 15 minutes.

3. Add adsorbent, shake 2 minutes, cool for 10 minutes, then centrifuge.

4. Collect the supernatant, recentrifuge if Fuller's earth was the adsorbent, add 0.5 ml of the supernatant to fluor solution.

5. Count two times.

6. Plot a standard curve of milliseconds/disintegration (Y) versus mμg (X) of steroid or determine the regression equation for this relationship. Use the graph or equation to determine the amount of steroid in the samples.

(1) Cortisol standard curves.

The source of CBG for cortisol standard curves was human plasma. Fuller's earth was used as the adsorbent. The range of the standard curve was 0-40 mμg. The specific activity of cortisol-1,2-T was 32 Ci/mM. The quantity of isotope used in preparing the CBG isotope solution was from 4.65 to 11.05 μCi/100ml. For ease of comparison all data were corrected to 6 μCi/100 ml.

Using 5% human plasma, and 60 mg Fuller's earth the equation for the standard curve (data from 8 determinations of the standard curve) was $Y = 0.0381 X + 1.203$. The standard deviation of the slope was 0.00214. Using the composite standard curve, the standard deviation of X, where X = 10 mμg and duplicate estimates of X are made, would be 4.57. The value 10 mμg was chosen because most human plasma samples are expected to contain an amount of cortisol in this range. For a single determination of the standard curve where the activity of the CBG isotope solution was

6 μCi/100ml the equation was Y = 0.0391 X + 1.359. The standard deviation
of the slope was 0.0026. Using this curve, where X = 10 mμg and duplicates
estimates of X are made, the standard deviation of X would be 2.44. This
standard curve is represented in Figure 9.
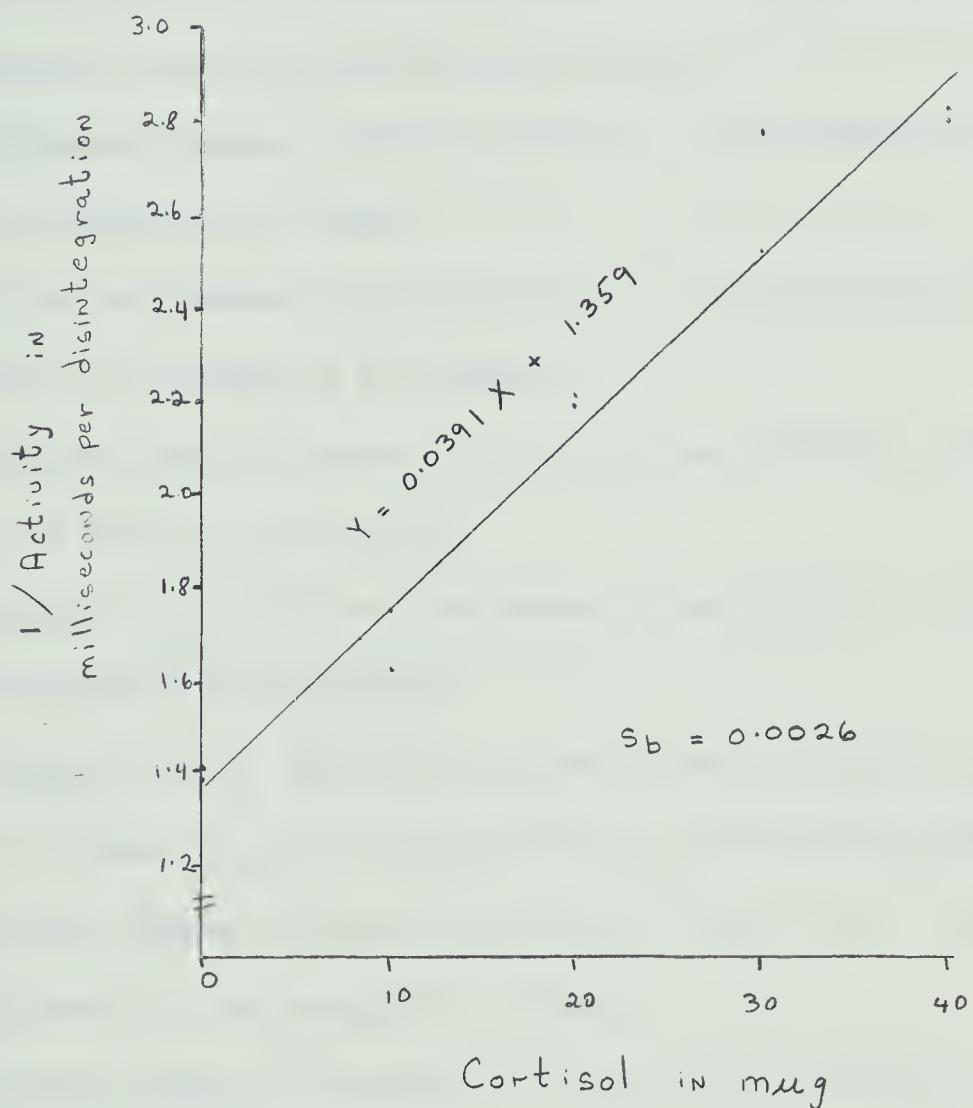
Figure 9.   Cortisol Standard Curve by CPB Radioassay.

(2) Corticosterone standard curves.

The source of CBG for the corticosterone standard curves was either mouse plasma or human plasma. Florisil or Fuller's earth was used as the adsorbent. Specific activity of corticosterone-1,2-T was 39.1 Ci/mM. The quantity of isotope used in each solution was from 3.82 to 14.46 μCi/100 ml. For ease of comparison all data were corrected to 6 μCi/100 ml. Several standard curves for corticosterone were prepared using the following materials:

1. 0.5% mouse plasma, ^{3}H-corticosterone, 8 mg Florisil (corticosterone in the range 0 to 5 mμg).

2. 0.5% mouse plasma, ^{3}H-corticosterone, 40 mg Florisil (corticosterone in the range 0 to 40 mμg).

3. 3% mouse plasma, ^{3}H-corticosterone, 8 mg Florisil (corticosterone in the range 0 to 20 mμg).

4. 1% human plasma, ^{3}H-corticosterone, 8 mg Florisil (corticosterone in the range 0 to 10 mμg).

5. 1% human plasma, ^{3}H-corticosterone, 8 mg Florisil (corticosterone in the range 0.5 to 2.5 mμg added to steroid-free serum).

6. 1% human plasma, ^{3}H-corticosterone, 60 mg Fuller's earth (corticosterone in the range 0 to 5 mμg).

7. 1% human plasma, ^{3}H-cortisol, 60 mg Fuller's earth (corticosterone in the range 0 to 10 mμg).

8. 2.5% human plasma, ^{3}H-corticosterone, 8 mg Florisil (corticosterone in the range 0 to 20 mμg).

In method 5, the steroid-free serum was obtained by diluting 1 volume of pooled human serum with 10 volumes of physiological saline and dialyzing the diluted serum against physiological saline for 66.5 hours. The saline was changed frequently during the dialysis period. After

dialysis the serum was concentrated to the original volume. 0.2 ml of
the serum was added to the standard quantity of corticosterone. Then the
standards were deproteinized and partitioned as described (section d,
section c (3)).

The results of the different determinations of the standard
curves are given in Table XX. The equation, the number of values (n)
used to determine the equation, and the standard deviation of the slope
(s_b) are given. For ease of comparison the standard deviation of the
slope is also expressed as the coefficient of variability (cv_b). Also
the standard deviation (s_x) of a 2 mμg sample is estimated assuming that
duplicate determinations are to be made. The value 2 mμg was chosen be-
cause most concentrations of corticosterone in human plasma samples are
expected to be in this range.

TABLE XX.

Corticosterone Standard Curves.

Method		Equation	n	s_b	cv_b	s_x [a]
1.		Y = 0.0619 X + 1.19	6	0.0061	9.9	0.42
2.		no slope				
3.		Y = 0.0079 X + 0.994	10	0.0011	14	2.5
4.	a	Y = 0.0732 X + 1.11	39	0.010	14	1.5
	b	Y = 0.0799 X + 1.17	10	0.0078	9.8	0.87
5.		Y = 0.0378 X + 0.914	6	0.0029	7.7	0.13
6.		Y = 0.374 X + 1.69	8	0.016	4.3	0.18
7.		Y = 0.0678 X + 1.31	10	0.0036	5.3	0.48
8.	a	Y = 0.0267 X + 1.03	18	0.0025	9.4	3.0
	b	Y = 0.0228 X + 1.09	10	0.0020	8.8	2.6

[a] For X = 2 mμg; where duplicate estimates are made.

It can be noted that method 6 gives the standard curve with the greatest slope. The variation in this curve is also the least. The standard deviation of X is also quite good. This latter value gives an estimate of the variation in conjunction with some consideration of the range since s_X will be low if variation of the slope is low and if the range is short. Increasing either of these factors will increase the value of s_X. If the coefficients of variability are about equal (eg. 1 and 4b) and the range of the standard curve is increased, the value of s_X will be increased (eg. s_X 4b $>$ s_X 1; range 4b $>$ range 1). Methods 1, 4, 5, 6 and 7 all are worthy of further consideration for the assay of corticosterone in plasma samples.

c. Interference in protein-binding radioassay.

(1) Interference of corticosterone in the assay of cortisol.

Using 5% human plasma CBG isotope solution and 60 mg of Fuller's earth a standard curve was prepared in the range 0 to 40 mμg of cortisol. It was used to assay samples containing various known amounts of cortisol and corticosterone. The samples were prepared from ethanol stock solutions of the steroids. The ethanol was evaporated prior to assay, then the samples were processed as described for the standards in the previous section. The results of the assays are shown in Table XXI. Interference was calculated by subtracting the known cortisol concentration from the found 'cortisol' concentration; it was expressed as mμg of interference per mμg of corticosterone. This was done by dividing the difference between the known and found values by the weight of corticosterone known to be in the sample.

TABLE XXI.

Interference of Corticosterone in Cortisol Assay[a].

Weight of Steroid in Sample (mμg)		Weight of 'Cortisol' by Radioassay (mμg)		Interference mμg/mμg B	
F	B	Sample 1	Sample 2	Sample 1	Sample 2
10	1	12.64	13.47	2.64	3.47
10	5	15.73	15.35	1.15	1.07
10	10	21.92	19.40	1.19	0.94
10	20	29.17	32.48	0.96	1.12
0	1	2.07	4.59	2.07	4.59
0	5	7.91	10.01	1.58	2.00
0	10	13.12	11.93	1.31	1.19
Average				1.81 ±	1.08 s

[a] Assay using 5% human plasma CBG isotope solution, ^{3}H-cortisol, 60 mg Fuller's earth, range 0 to 40 mμg.

(2) Interference of cortisol in assay of corticosterone.

Using 1% human plasma CBG isotope solution, ^{3}H-corticosterone, and 8 mg of Florisil a standard curve was prepared over the range 0 to 10 mμg. It was used to assay samples containing cortisol. Similarly, a standard curve using 1% human plasma CBG isotope solution, ^{3}H-corticosterone, and 60 mg of Fuller's earth covering the range 0 to 5 mμg was used to assay samples of cortisol. The interference of cortisol is shown in Table XXII.

TABLE XXII.

Interference of Cortisol in Corticosterone Assay.

Weight of Steroid in Sample (mµg)		Weight of 'Corticosterone' by Radioassay (mµg)		Interference mµg/mµg F	
F	B	Sample 1	Sample 2	Sample 1	Sample 2
10	0	6.95[a]	7.58 [a]	0.70	0.76
Average					0.72
5	0	5.22[b]	4.73[b]	1.04	0.95
Average					1.00

[a] Assay using 1% human plasma CBG isotope solution, ^{3}H-corticosterone, 8 mg Florisil, range 0 to 10 mµg.

[b] Assay using 1% human plasma CBG isotope solution, ^{3}H-corticosterone, 60 mg Fuller's earth, range 0 to 5 mµg.

The results of these interference studies indicate that human CBG offers no specificity for either cortisol or corticosterone; both are detected to approximately an equal extent.

(3) Carbon tetrachloride/water partitioning to separate corticosterone and cortisol.

In view of the lack of specificity for cortisol or corticosterone that had been demonstrated, it was necessary to attempt a separation. Carbon tetrachloride/water partitioning was the method tried.

The desired tritiated steroid was dissolved in 2 ml of distilled water and placed in a 30 ml separatory funnel. This solution was partitioned against 20 ml of carbon tetrachloride by shaking on a horizontal agitator. The two phases were then separated. The cortisol, being more polar than corticosterone, will favor the water phase while the corticosterone will prefer the carbon tetrachloride phase. The water phase was backwashed with 20 ml of carbon tetrachloride by mechanical shaking;

this removed carried-over corticosterone. Similarly, the initial carbon tetrachloride phase was backwashed with 2 ml of distilled water. In each case, the washings were discarded. A one quarter aliquot of each of the desired phases was taken for counting. The water (0.5 ml) was added to 10 ml of Bray's solution for counting to determine the recovery of cortisol and the crossover of corticosterone. The carbon tetrachloride (5 ml) was evaporated in the counting vial prior to addition of fluor. When this means of separation was used in conjunction with the assay procedure the initial radioactive steroids were not added; the endogenous steroid only was determined. Also a one half aliquot of each of the desired phases was taken and evaporated in a centrifuge tube. The water was evaporated at reduced pressure in a freeze drying apparatus. The carbon tetrachloride was evaporated at atmospheric pressure while immersed in a water bath at a temperature not greater than $45^{\circ}C$. The steroid was washed to the tip of the centrifuge tube with two 1 ml washes of carbon tetrachloride. When a nitrogen stream was used in place of an air stream for evaporation, no difference was observed in the assay results. The different evaporation techniques were necessary because of the difference in volume and volatility of the two solvents. Batches of eight samples were prepared simultaneously in each assay using this procedure.

(a) <u>Time of shaking</u>.

The above procedure was carried out without the backwash steps to determine the length of shaking time necessary. The results are shown in Table XXIII.

TABLE XXIII.

Effect of Shaking Time on Partitioning of Cortisol and Corticosterone.

Time (min)	Water Phase		Carbon Tetrachloride Phase	
	F Recovery	B Crossover	B Recovery	F Crossover
2	85.4 %	16.7 %	83.3 %	14.6 %
5	83.5 %	16.7 %	83.3 %	16.5 %

Two minutes shaking was chosen as the better time because longer shaking gave decreased recovery of cortisol and increased crossover of cortisol into carbon tetrachloride.

(b) Recovery of corticosterone and cortisol after carbon tetra-chloride/water partitioning.

The procedure was used as outlined above with the backwash steps included. Replicates were performed on different days (Table XXIV).

TABLE XXIV.

Separation[a] of Corticosterone and Cortisol by Carbon Tetrachloride/Water Partitioning.

Replicate	Water Phase		Carbon Tetrachloride Phase	
	F Recovery	B Crossover	B Recovery	F Crossover
1	74.1 ± 2.5[d]	15.6 ± 1.2[c]	48.5 ± 4.3[d]	6.3 ± 0.6[d]
2	74.1 ± 1.0[d]	6.9 ± 0.3[d]	76.2 ± 2.4[d]	7.5 ± 0.4[d]
3		8.6 ± 0.3[d]	62.0 ± 1.8[d]	
4		12.6 ± 1.2[d]	35.4 ± 2.4[d]	
5[e]	68.0 ± 0.3[b]	13.9 ± 1.3[b]	53.5 ± 0.1[b]	5.7 ± 0.1[b]

[a] Expressed as average % ± standard deviation.

[b-c-d] Number of samples equals 2, 3 or 4 respectively.

[e] Steroids dissolved in 10% aqueous serum rather than water.

The recovery of cortisol in water was reproducible from day to day (cortisol recovery 74.1 ± 1.8 (s)%; cortisol crossover 6.9 ± 0.8 (s) %); however
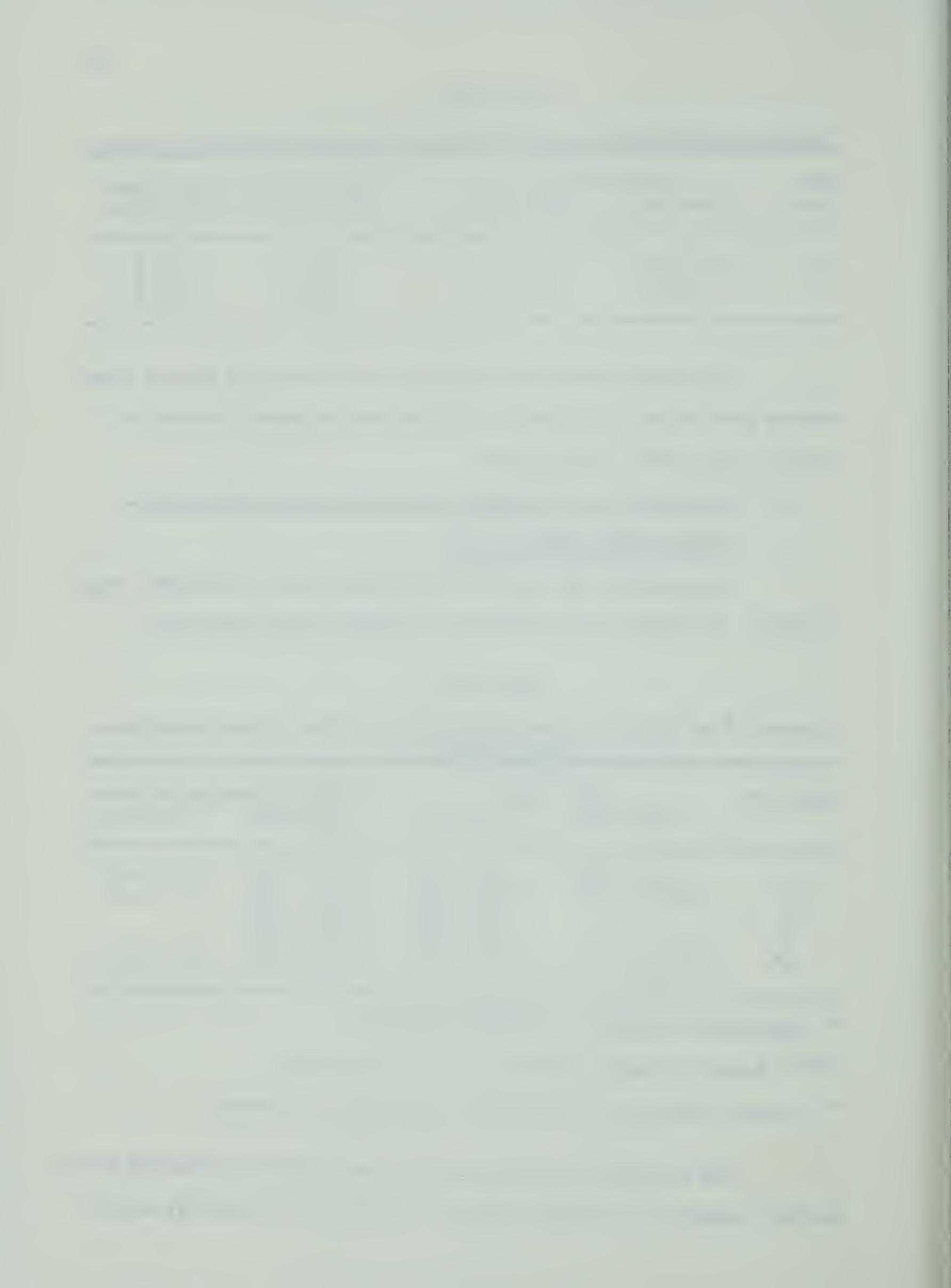
the recovery of corticosterone though reproducible on any given day was
not reproducible from one day to another. For this reason daily recovery
indicators are required. It can be seen that complete separation of the
two steroids was not attained. The presence of serum in the aqueous phase
reduced the recovery of cortisol.

    d.   Recovery of cortisol and corticosterone from plasma.

       Having established a means to assay cortisol and corticosterone
and also having established a means to separate the two from each other,
it was necessary to recover the steroids from plasma. Since CBG (the
assay protein) is normally present in plasma, it was necessary to remove
it from the sample prior to assay. A comparative study on the deprotein-
ization of the samples was attempted using 95% and absolute alcohol. For
testing the adequacy of recovering cortisol and corticosterone, serum
samples mixed with radioactive steroids were used. In the first case
(95% alcohol), 0.2 ml of serum was mixed with 0.1 ml of an aqueous sol-
ution of ^{3}H-cortisol or ^{3}H-corticosterone to give the sample. In the
second case (absolute alcohol), the desired quantity of the ^{3}H-cortisol
or ^{3}H-corticosterone in ethanol solution was placed in a centrifuge tube,
the alcohol was evaporated, 0.2 ml of serum was added, and the sample was
mixed thoroughly. The serum proteins were precipitated with 1 ml of al-
cohol (95% or absolute) by mechanical mixing for 2 minutes followed by
centrifugation. The supernatant was removed and the last step was re-
peated on the precipitate. The combined alcoholic supernatants were
evaporated in a scintillation vial prior to addition of fluor and count-
ing. Where this procedure was used in conjunction with the assay the com-
bined supernatants were evaporated in a test tube by use of a freeze dry-
ing apparatus. These residues were partitioned as previously described.
The results shown in Table XXV were determined in experiments on two dif-

ferent days. Since reproducibility on these two days was good the results from the two experiments were averaged.

TABLE XXV.

Recovery[a] of Cortisol and Corticosterone from Serum by Alcohol Precipitation of Proteins.

Steroid	95% alcohol	n	Absolute alcohol	n
F	69.2 ± 11.8	12	67.5 ± 7.8	9
B	89.2 ± 7.3	12	60.6 ± 8.3	8

[a] Average % recovery ± standard deviation.

Because a better recovery was observed when 95% alcohol was used as the precipitating reagent, it was decided to adopt this procedure for the assay of plasma samples.

e. Application of method.

The method of deproteinization, partitioning and assay could now be combined and this total method could be used for the assay of plasma samples. A new standard curve was prepared with each run.

(1) Procedure.

Plasma samples (0.2 ml) were deproteinized as previously described (section d). The cortisol and corticosterone were separated as previously indicated (c (3) (b)). For cortisol determinations the residues from the water phases were assayed using 5% human plasma CBG isotope solution, ^{3}H-cortisol and 60 mg Fuller's earth. For corticosterone determinations, the residues from the carbon tetrachloride phases were assayed using one of the following methods:

A.   1% human plasma CBG isotope solution, ^{3}H-corticosterone, 8 mg Florisil.

B.   1% human plasma CBG isotope solution, ^{3}H-corticosterone, 8 mg Florisil, standards in steroid-free serum.

C. 1% human plasma CBG isotope solution, ^{3}H-corticosterone, 60 mg Fuller's earth.

D. 1% human plasma CBG isotope solution, ^{3}H-cortisol, 60 mg Fuller's earth.

E. 5% human plasma CBG isotope solution, ^{3}H-cortisol, 60 mg Fuller's earth.

The method using mouse plasma was not used because of the difficulty in obtaining sufficient mouse plasma. Method E was tried because of the potential advantage of speed if only one standard curve and CBG isotope solution was needed to assay both cortisol and corticosterone. Since the interference study had shown that the two steroids were detected to about equal extents it seemed feasible that both could be determined with the same CBG isotope solution.

Correction for recovery was effected in two ways. Since recovery was reproducible within a run, recovery factors for each sample were not deemed necessary; however, since recovery was different from day to day, at least for corticosterone, a daily recovery factor was needed.

(a) Recovery correction by isotope yield determination.

With each batch of samples two yield determination standards were included. One contained a measured amount of cortisol-1,2-T (2 Ci/mM) in 0.2 ml of plasma, while the other contained corticosterone-1,2-T (1 Ci/mM) in the same volume of plasma. These standards were taken through the protein precipitation and partitioning steps in parallel with the samples. These standards were processed no further except for counting of radioactivity. Each standard yielded two counting samples, one for recovery and one for crossover. The fractional recovery and crossover were calculated by comparison to the initial radioactivity added. Because the normal concentration of cortisol in human plasma is much higher than that of corticosterone, crossover is less important in the determination of cortisol.

In the case of corticosterone correction for crossover is important in order to avoid over estimating the corticosterone concentration of a sample. The value of cortisol (corrected for recovery) is multiplied by the fraction crossover of cortisol into carbon tetrachloride. This figure is subtracted from the recovery corrected amount of corticosterone to remove the contribution of cortisol. In physiological states where the ratio of cortisol to corticosterone is reversed, this procedure would be used to find the cortisol (corrected) concentration in the sample.

(b) <u>Recovery correction by internal standard method.</u>

In this procedure a known concentration (usually 10 mμg) of cortisol or corticosterone was added to 0.2 ml of plasma. A sample containing a known amount of added cortisol and another sample containing a known amount of added corticosterone are prepared as well as a sample of the same plasma containing no added steroid. These three samples are processed in parallel with the other samples. From the difference between the amount of steroid detected in the plasma only sample and the sample with added steroid, the recovery can be determined.

In both cases, the recovery corrected amount of steroid was calculated by dividing the uncorrected weight of detected steroid by the fractional recovery factor. The results are expressed in mμg/0.1 ml plasma and are numerically equal to concentration in μg/100 ml plasma.

(2) <u>Interference.</u>

In order to determine whether corticosterone was interfering to a detectable extent in the total method for assay of cortisol, several sets of plasma samples were assayed using the previously described procedure. The set of samples consisted of several aliquots of the same sample, some with and some without additional corticosterone. Also, several samples (no plasma) of cortisol and corticosterone and a mixture

of the two together were processed. The samples in each set were compared
to see if the additional corticosterone caused a significant difference in
the results of the assay. The 10% level was used to judge the significance
of differences; this gave a better chance of detecting any interference.
Table XXVI shows the various results obtained.

TABLE XXVI.

Interference of Corticosterone in Competitive Protein Binding Radio-assay of Cortisol[a].

Sample	Added Steroid (μg/100 ml)		'Cortisol' found (μg/100 ml)		n	Interference
	F	B				
1. EtOH solution of steroid[b]	10	0	7.18,	9.06	2	no[d]
	10	1	7.19,	10.27	2	
	10	10	8.26,	10.81	2	
2. EtOH solution of steroid[b]	0	10	2.13		1	yes 0.21 μg/μg B
3. Plasma[c]	0	0	9.11 ± 2.96 s		7	no[e]
	0	10	10.91 ± 2.32 s		6	
4. Plasma[c]	0	0	10.75 ± 1.98 s		8	yes[f]
	0	10	13.85 ± 1.97 s		7	0.31 μg/μg B
5. Plasma[c]	0	0	12.03 ± 2.04 s		4	no[g]
	0	10	10.54 ± 1.67 s		4	
6. Plasma[c]	0	0	11.94 ± 2.36 s		3	no[h]
	0	5	16.82 ± 3.10 s		2	

[a] Assay using 5% human plasma CBG isotope solution, ^{3}H-cortisol, 60 mg Fuller's earth, subsequent to protein precipitation and CCl_4/H_2O partitioning.

[b] Recovery correction by yield determination method.

[c] Recovery correction by internal standard method.

[d] F < 1; not significant.

[e] t = 1.20; not significant.

[f] t = 3.03; significant (P < 0.01).

[g] t = 1.13; not significant.

[h] t = 2.03; not significant.

(3) <u>Comments on application of method to determination of corti-</u>

<u>costerone.</u>

When methods A, C and D for the determination of corticosterone were applied to the carbon tetrachloride phase after the partitioning of plasma samples, the quantities of corticosterone detected were negative. That is the values of milliseconds/disintegration for plasma samples fell below that for the zero mμg standard. This elevated activity in these samples could be due either to a decrease in adsorption of free steroid or to an increase in the protein-binding of steroid. An increase in binding of steroid to protein could occur if additional binding protein, other than the CBG isotope solution, were carried over in an undenatured form from the initial sample to the assay sample. Ethanol precipitation did not completely remove protein as shown by a positive ninhydrin test[a]. When the ethanol supernatant was treated with an excess of ammonium sulfate and filtered, the supernatant was found to be free of protein as shown by the ninhydrin test. This ethanol was then evaporated and partitioned. When the carbon tetrachloride phase of this sample was assayed using method A, the value of milliseconds/disintegration was still lower than that for the zero point on the standard curve.

Methods E and B did give positive values for corticosterone as determined in the carbon tetrachloride phase after protein precipitation and partitioning. They were thus judged to be most suitable for the actual determination of this steroid.

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[a] Ninhydrin test: Ninhydrin (0.5% in butanol) Nutritional Biochemicals spray reagent was used. Some of the ethanol supernatant was dried on filter paper, then sprayed with ninhydrin reagent, dried and heated to 80ºC several minutes. A pink-purple colour indicated the presence of protein.
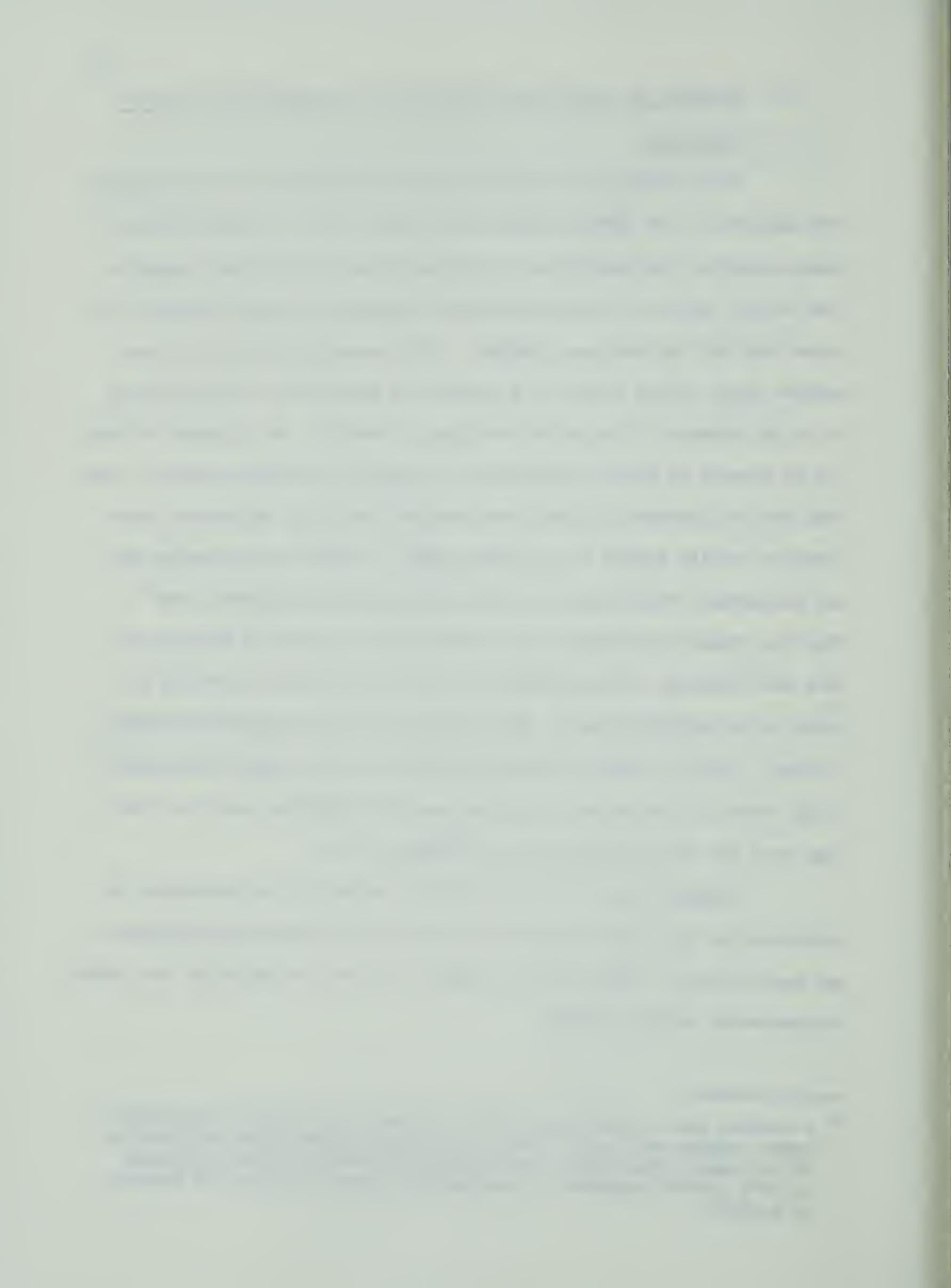
(4) Precision.

The precision of the total method was determined by calculation of the standard deviation of replicate determinations on aliquots of several samples of plasma. Table XXVII demonstrates the various results observed.

TABLE XXVII.

Precision of the Competitive Protein Binding Radioassay[a].

Steroid	Mean Concentration (µg/100 ml)	Number of Replicates	Standard Deviation
F	9.11	7	2.96
	10.75	8	1.98
	11.94	3	2.36
	12.03	4	2.04
	19.11	7	4.31
	20.76	7	1.20
	22.04	3	0.83
B	4.96	7	2.77
	7.62	6	2.20

[a] Assay using 5% human plasma CBG isotope solution, ^{3}H-cortisol, 60 mg Fuller's earth, subsequent to protein precipitation and partitioning. Recovery correction by internal standard method for cortisol and yield determination method for corticosterone.

(5) Accuracy.

The accuracy of the method was determined using aliquots of four different plasma samples. The concentration of cortisol in each sample was determined, then 10 µg/100 ml of cortisol was added and the concentration was again determined. Several replicate determinations were made in each case. When the internal standard method of recovery correction was used, the additional increment detected was 8.24, 9.12, 11.83 and 12.46 µg/100 ml; compared to 8.40, 9.02, 15.94 and 4.57 µg/100 ml using the yield determination method of recovery correction. It was judged that the
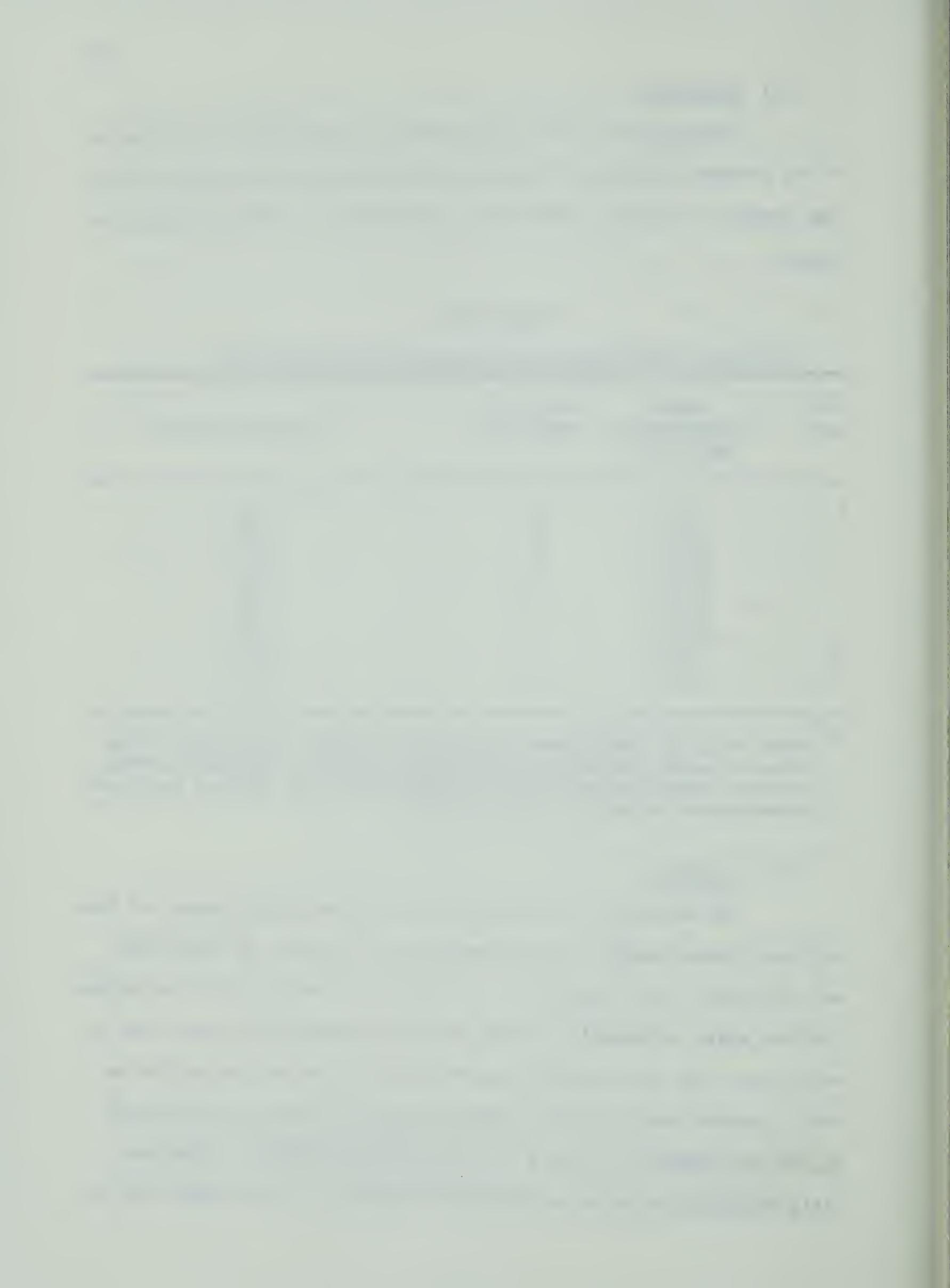
internal standard method gave more accurate results.

(6) Estimate of detectable differences.

Using 5% human plasma CBG isotope solution, ^{3}H-cortisol and 60 mg Fuller's earth method for determining cortisol in plasma (0.2 ml) the estimated standard deviation was 2.65 µg/100 ml. The alpha value, which indicates the level of significance or the probability that a difference does not exist, was set at 95 and 90%. The beta value, which indicates the probability that a difference does exist, was set at 80%. The approximation of Cochran and Cox (124) was used to determine the detectable differences (Table XXVIII) with 2 to 5 replicate determinations on a sample at the two levels of significance.

TABLE XXVIII.

Estimate of Detectable Differences.

Number of Replicate Determinations	Detectable Differences (µg/100 ml)	
	95% (α) 80% (β)	90% (α) 80% (β)
2	37.32	20.38
3	11.61	8.61
4	7.80	6.24
5	6.23	5.15

(7) Normal values.

The method of determination of cortisol was applied to three samples of normal human plasma (male subject) which were drawn in the morning (10:00 AM to 12:30 noon). These samples were all drawn from the same subject but were drawn on different days. The concentration of cortisol was found to be 11.71 ± 1.50 (s) µg/100 ml (n = 3) when recovery correction was done by the internal standard method, and 12.24 ± 7.96 (s) µg/100 ml (n = 3) when recovery correction was done by the yield
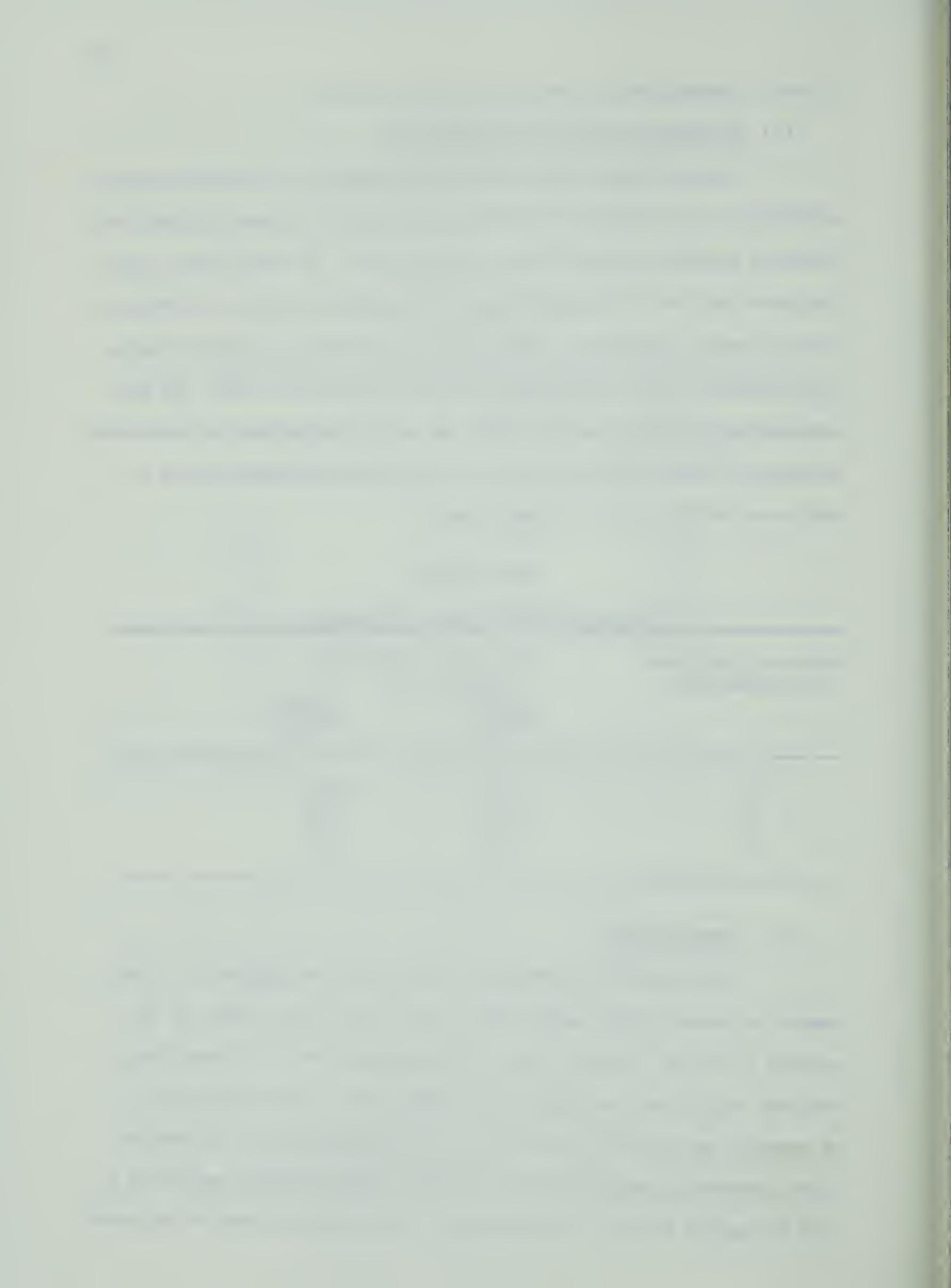
determination method. When cortisol determinations were made on pooled
human serum the concentration was found to be 13.79 µg/100 ml (recovery
correction by yield determination method). When method E was used for
corticosterone determination the level (12:30 noon) was found to be 4.96
µg/100 ml (recovery correction by yield determination method). When
method B was used to determine corticosterone levels (10:15 AM) the con-
centration was found to be 1.84 µg/100 ml. In the latter case no recov-
ery correction was needed because standards and samples were processed
in parallel through all steps of protein precipitation, partitioning and
final assay.

DISCUSSION

The assessment of adrenal activity by the determination of levels of adrenocortical steroid hormones is useful both clinically and investigationally. Methods for the determination of corticoids initially were applied to urine because of the difficulty in determining the very small amounts of these steroids found in blood. The disadvantage of all steroid assays on urine is the lag between the change in gland activity and detection of the change in the urine (125). Blood levels, on the other hand, are a much more rapid indicator of changes in adrenal activity; for example, an increase in corticoid levels after intravenous administration of ACTH can be detected within 15 to 30 minutes, while a rapid drop occurs when the ACTH is stopped (3). Blood levels are a more accurate reflection of adrenal activity since the determination is made closer to the source of the hormone without interposing renal factors (126). Also, a better idea of the corticosteroid available for transport to tissues is obtained (127). A further advantage of assay of adrenal hormones in blood, rather than urine is that since the blood picture is simpler than that of urine, the assay is easier to evaluate and is less liable to interferences (127). Blood levels of corticoids fluctuate rapidly so for a composite picture of activity a 24 hour urine determination may be preferable (127). Hormone activity may not be represented accurately by total blood levels because factors other than concentration, such as protein binding, may act to limit the significance of a change in steroid level (2). Most of the spectrophotometric methods of analysis require large volumes of plasma for the determination of corticosteroids. A method which uses small volumes of plasma is desirable since repeated determinations of corticoids (eg. diurnal variation, ACTH stimulation, dexamethasone suppression) are much more meaningful (1, 128). Methods using small plasma volumes are also useful for the study

of adrenocortical dynamics (137). If a small sample is used routinely, a larger sample can be employed to increase accuracy where a low value is anticipated (eg. dexamethasone suppression). Furthermore it is an easy matter to store small samples for determination at a later date in order to check data. In addition, several different hormone determinations can be made when only a small sample of plasma is required for each.

A method which would individually determine cortisol and corticosterone would be desirable. It has been shown that administration of ACTH to humans in some cases causes a change in the cortisol to corticosterone ratio (129). Because of the lack of methods for determination of cortisol and corticosterone individually, little investigation of the physiological significance of a changing cortisol/corticosterone ratio has been done (130); however it is known that changes in corticosterone secretion do not always parallel changes in cortisol. In the rabbit prolonged ACTH administration causes a change in corticosterone/cortisol ratio from 20:1 to unity (131, 132).

Separation procedures were employed with a view to rapidity at the expense of quantitative recovery. This necessitates the use of recovery correction factors resulting in some loss of precision. The separation of cortisol and corticosterone by partitioning between water and carbon tetrachloride has been used previously (40). In the present study separation was checked by use of radioactive steroids. This approach indicated that separation was not complete. The proportion of cortisol/corticosterone in the carbon tetrachloride was about 6/100 and the proportion of corticosterone/cortisol in the water was about 8/100 (Table X). The carbon tetrachloride phase will contain other steroids than corticosterone. It will take up 11-deoxycortisol just as avidly as corticosterone (partition coefficient water/carbon tetrachloride:

corticosterone 0.75, 11-deoxycortisol 0.74) (2). Bowman (109) reported

that 80% of 11-deoxycortisol will be removed by extraction of plasma

with 10 volumes of carbon tetrachloride. The normal levels of 11-deoxy-

cortisol are low (0-2 µg/100 ml) (133), but may be significant when com-

pared to the low concentrations of corticosterone in plasma (1 µg/100 ml).

11-Deoxycortisol is detected quite strongly in the CPB method of assay

(106, 108), but to a very little extent in fluorometric assay (40); there-

fore, in the former type of method the 'corticosterone' value would be

inflated by the presence of 11-deoxycortisol. This would be especially

important in cases of pharmacological blockade of 11-hydroxylation with

Metopirone, where levels of 11-deoxycortisol rise sharply. Rudd (41)

has shown that pregnanetriol and pregnanetriolone will be present in the

carbon tetrachloride phase. The blood levels of these two steroids are

normally low, but may be elevated in adrenal hyperplasia where 21-hydrox-

ylation is reduced. These two compounds would provide interference in a

fluorometric assay (41) but not in a CPB assay (108). Other steroids

which would favour the carbon tetrachloride phase, judged from partition

coefficient data (2), are testosterone, progesterone and androstenedione.

The normal levels of these steroids are 0.5 µg/100 ml for testosterone,

0.17 µg/100 ml for androstenedione, and less than 2 µg/100 ml for pro-

gesterone in non-pregnant females and less than 0.8 µg/100 ml in males

(134, 135, 106). These levels are significant relative to corticosterone

concentration. The two androgens would not interfere in a CPB method;

however progesterone would inflate the values of corticosterone estimated

from the carbon tetrachloride fraction (106). Kolanowski (110) suggests

that testosterone is strongly detected in the CPB method so it may pose

an interference problem. Testosterone and progesterone would not likely

interfere in a fluorometric assay because of the high relative fluor-

escence of corticosterone (39).

Attempts at removal of cholesterol from plasma or plasma extracts were not successful because very considerable loss of corticoids occurred when other partitioning steps were coupled with the carbon tetrachloride/water partitioning. Matsumara (39) had felt this would be the case. Of the many fluorometric methods available most do not make any effort to remove cholesterol from plasma except to wash the plasma with petroleum ether or iso-octane, or partition the extract, after evaporation and solution in aqueous solvents, against hexane, ligroine or skellysolve. The main concern is to remove the interfering fluorogens, which are as yet unidentified (136). It is quite possible that cholesterol does not make up a substantial part of this interference and our concern with cholesterol may have been undue. It has been shown that background fluorescence does not bear any relationship to cholesterol levels (137). Nielsen (37) reported that although cholesterol added to methylene chloride extracts significantly increased fluorometric estimates of corticoids, no cholesterol could be detected in the methylene chloride extracts of plasma. He suggested cholesterol was not extracted into methylene chloride perhaps due to protein binding. Nielsen did not indicate how cholesterol was determined in the extracts; it is possible our isotope method was more sensitive.

The fluorescence of corticosterone was greater in 60% sulfuric acid-ethanol than in 75% sulfuric acid-ethanol, while the opposite was true for cortisol. The 60% sulfuric acid-ethanol mixture was found to give most reliable results, as has been stated by other investigators (39). In establishing the calculation method of resolving cortisol and corticosterone Matsumara showed the 'a' and 'b' ratios to be reproducible for one concentration of cortisol and corticosterone (0.5 μg/tube).

They verified their method using a sample of known and equal concentrations of cortisol and corticosterone. Results of the present study showed that the 'a' and 'b' ratios were not constant at all concentrations of cortisol and corticosterone and in fact varied with concentration.

The useful range of the fluorescence standard curve was 0.005-1 μg/100 ml. If we choose the sample size such that normal values fall in the middle of this range we will allow for detection of an increase or a decrease in plasma corticoid levels. If we assume 50% recovery up to the fluorometric assay step 5 ml of normal plasma would give a concentration of 0.02 μg corticosterone per ml and 0.2 μg cortisol per ml in the final fluorometric sample. This would allow for a fifty- and five-fold increase in corticosterone and cortisol respectively without going out of range. Stress and Cushing's syndrome have been reported to give approximately a threefold increase in cortisol concentration (138, 139) while ACTH produces a three- to sixfold increase (37, 139).

In many fluorometric methods the steroid is extracted directly from the solvent extract into the fluorescence reagent, while in the present study the solvent was evaporated then the fluorescence reagent was added. Although the latter method is more time consuming it would appear to be the more reliable since the different steroids (cortisol and corticosterone) may be extracted into the fluorescence reagent with different efficiencies. The extraction of steroid into reagent has been found to give erratic results in a spectrophotometric method which employed a reagent made up largely of sulfuric acid (126); however the extraction may lend some specificity.

Adsorption of ^{3}H-cortisol by Fuller's earth is constant for weights from 40 - 120 mg (Table XIV). For this reason we felt the ac-

curacy of measuring Fuller's earth was not very critical, unlike Bowman

(109) who found the 'spoon' method of measuring to be unsatisfactory.

Studies of the removal of free steroid from solution (Tables

XIV, XVII)showed that complete adsorption of ^{3}H-cortisol by Fuller's

earth and ^{3}H-corticosterone by Florisil was not achieved; adsorption of

cortisol by 60 mg of Fuller's earth was approximately 84% and of corti-

costerone by 8 - 10 mg of Florisil was about 82%. This means that there

will be residual free activity remaining in the 'protein-bound' phase

when separation of bound and free steroid is attempted by use of ad-

sorbents. This residual free activity will decrease the relative signif-

icance of a small change in activity thus causing a decrease in sensitiv-

ity and a decrease in the ability to detect differences by this radioassay.

The decreased adsorption of cortisol by 120 mg of Fuller's earth

at 1 mμg level of cortisol suggests that at low concentrations of sample

cortisol where little ^{3}H-cortisol is displaced there will be a dispropor-

tionate increase in the activity of the 'protein-bound' fraction, and

consequently a lower milliseconds/disintegration value than would be

anticipated from a linear expression of the standard curve. This means

that low values of cortisol would be underestimated. This effect was not

however observed perhaps because the amount of free cortisol in the CBG

isotope solution exceeded 1 mμg/ml. Decreased adsorption of corticost-

erone by Florisil at the 1 mμg level was not demonstrated, thus would

not explain the negative values obtained for corticosterone plasma ex-

tracts.

Fuller's earth and Florisil are not without effect on protein-

bound steroid (Tables XVIII, XIX) as shown by the fact that the percent-

age protein-bound steroid is not constant at all weights of adsorbent.

From Table XVIII it can be seen that at high concentrations (120 mg) of

Fuller's earth some of the protein-bound cortisol is removed from solution in addition to the free cortisol as shown by a decrease in percentage protein-bound. For Florisil (Table XIX) protein binding of corticosterone is interfered with by all amounts of Florisil; no high plateau of protein binding results. At $4^{\circ}C$ the half-dissociation time of cortisol and CBG is 25 minutes (98). The exposure of adsorbent to CBG-steroid complex is about 15 minutes. This means that there will be some dissociation of the complex during adsorbent exposure. When a large concentration of adsorbent is present the dissociated steroid has more chance to be removed by adsorption prior to reassociation with the protein, while at lower adsorbent concentrations the steroid and the protein reassociate before the removal by adsorbent takes place. This could explain the fall in percentage protein-bound steroid at higher amounts of adsorbent.

For assay purposes it is desirable to chose an amount of adsorbent such that small changes in adsorbent weight will not cause a marked change in protein binding, but there must also be a substantial amount of protein binding so that changes in sample steroid weight will be easily detected in terms of a marked change in steroid binding. For Fuller's earth this was possible, but not for Florisil. Murphy (106) found maximum difference between adsorption by Fuller's earth from aqueous solution of ^{3}H-cortisol and protein solution of ^{3}H-cortisol in the range of 15 - 60 mg; our results confirmed this as we found the maximum difference (which we referred to a % protein-bound steroid) in the range 20 - 70 mg of Fuller's earth. We differed from Murphy in the choice of optimum adsorbent concentration. Murphy chose the minimum amount to give maximum adsorption of free steroid, while we chose an amount in the middle of a range of adsorbent weight giving maximum adsorption of free cortisol and also the middle of a range of weights

giving maximum protein binding. The latter approach would give more precise results since slight errors in measuring adsorbent would be of little significance in the assay; with Murphy's choice errors in adsorbent weight could have profound effects on the results of the assay.

The effect of dilution of CBG is to decrease the number of CBG binding sites available so that small amounts of sample steroid are more significant relative to the total number of sites available; that is the sensitivity of the assay is increased. On the other hand the useful range of the assay is decreased.

The theoretical limit of useful range is reached when sufficient steroid sample has been added to dilute the specific activity of the protein-steroid complex to a point indistinguishable from zero, but in practice zero activity is never reached because of the presence of unadsorbed free activity in the supernatant. Hence the practical limit of the range is reached when further additions of steroid cause no further reduction in activity of the supernatant. The standard curve is seen to lose its slope and become flat; it is no longer linear when the range is exceeded.

The degree of steroid saturation of the CBG in the CBG isotope solution will have an effect on the sensitivity of the radioassay. For maximum sensitivity one needs to just saturate the CBG. Failure to saturate the CBG will decrease the sensitivity of the assay because small quantities of sample steroid will be bound to the CBG with little or no displacement of labelled steroid. When the sample size is sufficiently large to saturate the unoccupied sites on the CBG and displace labelled steroid, the sample will be detected. If the CBG in the isotope solution is oversaturated (i.e. all binding sites are occupied and there is still excess steroid present in an unbound form) by an excess

of labelled steroid, the effect will be to increase the specific activity
of the protein complex. This increased specific activity should increase
the sensitivity of the assay, but because a large pool of free steroid
(radioactive and nonradioactive) is also present in the CBG isotope sol-
ution, the addition of a small quantity of sample steroid will have less
significance so the new equilibrium which results will produce only a
slight decrease in the radioactivity of the CBG. Thus adding more label-
led steroid than necessary to saturate the CBG, will decrease the sensi-
tivity of the assay.

It is difficult to judge saturation of the CBG in the isotope
solution. It is possible to estimate the amount of added steroid re-
quired to saturate the CBG by considering the cortisol binding capacity
(CBC) of the standard plasma, its endogenous steroid concentration and
the specific activity of the labelling steroid. For example, for corti-
sol using 5% human plasma CBG isotope solution (6 μCi/100 ml) for assay,
we can estimate whether the CBG in this solution was saturated by the
following calculations: 5% human plasma contains 0.05 ml plasma/ml solu-
tion; CBC of human plasma is 22 μg/100 ml = 11 mμg/0.05 ml; endogenous
cortisol is 10 μg/100 ml = 5 mμg/0.05 ml; specific activity of ^{3}H-cort-
isol is 32 Ci/mM = 11.35 mμg/μCi; cortisol added with label = 11.35 X
$\frac{6}{100}$ = 0.68 mμg/ml; total cortisol in CBG isotope solution = 5.68 mμg/ml.
Since 11 mμg/0.05 ml are required to saturate the CBG, this CBG isotope
solution is not saturated. It would require 11 - 5.68 = 5.34 mμg of
cortisol to saturate the CBG, thus samples containing less than 5.34
mμg of cortisol would not be detected. Similar calculations for 1%
human plasma CBG isotope solution (6 μCi/100 ml) indicate it is approx-
imately saturated. This calculation method of judging saturation is
not ideal. It is likely to be in error because estimates of CBC and

endogenous cortisol concentration were used in the calculation.

Another way to judge saturation of the CBG in the CBG isotope solution, is to compare the activity of the CBG isotope solution before and after adding adsorbent but without addition of sample steroid. Since the adsorbent removes free steroid, the two activities should be the same if the solution is unsaturated or just saturated, and the 'after adsorbent' activity should be lower if the solution is oversaturated. Using this criteria all CBG isotope solutions (5% human plasma CBG isotope solution 4.65 - 11.05 μCi/100 ml, and 1% human plasma CBG isotope solution 3.8 - 14.5 μCi/100 ml) were unsaturated. This criteria for judging saturation of CBG can be criticized on the grounds that few proteins have sufficient affinity for steroids such that all steroid is bound when the protein is unsaturated (111); thus even if the CBG is unsaturated some free steroid will be present in the solution. This suggests that a difference in CBG activity before and after treatment with adsorbent does not imply oversaturation of the CBG.

It can be seen that it is difficult to determine whether the CBG is just saturated, so judging the amount of steroid to add while labelling the CBG, must be empirical. Inevitably sensitivity will not be maximal.

Removal of corticosterone by Fuller's earth is greater than the removal of cortisol (106). If we compare the standard curves derived using 0 - 10 mμg of corticosterone, 1% human plasma isotope solution, 60 mg of Fuller's earth and either ^{3}H-cortisol ($Y = 0.0678\ X + 1.31$) or ^{3}H-corticosterone ($Y = 0.374\ X + 1.69$) we can see that the slope of the latter was approximately 5 times greater indicating a much greater change in activity per unit of corticosterone added as sample. This could arise either due to a more intense removal of ^{3}H-corticosterone

by Fuller's earth or due to a greater affinity of CBG for cortisol resulting in it being more difficult for corticosterone to displace cortisol than for corticosterone to replace corticosterone. In either case the curve prepared using ^{3}H-corticosterone as labelling agent would be more sensitive and precise.

Murphy attributes specificity to the use of adsorbents (106). In the present study this was not demonstrated. Using 1% human plasma, ^{3}H-corticosterone, and 8 mg of Florisil for the assay of corticosterone, we found that cortisol interfered to the extent of 0.72 mµg/mµg; while using the same system except using Fuller's earth (60 mg) in place of Florisil for the assay of corticosterone, cortisol was found to interfere to the extent of 1 mµg/mµg. Using the system of 5% human plasma, ^{3}H-cortisol and 60 mg of Fuller's earth to assay cortisol, corticosterone was found to interfere to the extent of 1.81 mµg/mµg of corticosterone. It seems reasonable that this effect would be observed since the measurement of steroid is dependent only on the decrease in activity observed. The adsorbent role is to remove free steroid after the stage of equilibration of sample steroid with CBG. The effect of the adsorbent on the assay will be dependent only on the form of radioactive steroid used, since adsorbent removal of any other steroid will go undetected. The source of the protein used determines specificity. Since human CBG has approximately equal affinity for cortisol and corticosterone (106), both will be detected and there will be no specificity for one or the other. ^{3}H-cortisol on the CBG will be displaced by any steroid with equal or greater affinity for the CBG and the free ^{3}H-cortisol will be removed from solution by adsorption depending on the affinity of the adsorbent for cortisol. The different adsorbents will affect the slope of the standard curve and thus the sensitivity and precision of the assay

but will not affect the specificity of the assay.

Bearing in mind this lack of specificity for cortisol and corticosterone some means of separation was necessary in order to individually estimate them. Carbon tetrachloride/water partitioning was chosen because of rapidity, ease of handling a large number of samples, and simplicity; although some sacrifice of specificity was inevitable. The separation methods have been discussed earlier.

The method of deproteinization was shown to be incomplete but this may not be important since it is the destruction of the specific binding protein that is important and not the destruction of all protein in the sample (111).

Recovery correction factors were necessary because extraction was not complete at the carbon tetrachloride/water partitioning step. Since recovery of corticosterone was shown to vary from day to day it was necessary to include standards with each run. Of the two approaches used the internal standard method gave more reliable results. This is probably because it corrects for losses including any that occur in the assay stage, whereas the yield determination method corrects for losses only up to but not including the assay step. This may be important because as shown before deproteinization was not complete and this small amount of protein carried to the final assay step may alter the efficiency of the detection of the steroid in the CBG radioassay. Any other non-steroid material carried to the final assay stage might likewise alter the detection efficiency for the desired steroid. In most cases the internal standard factor was lower than the yield factor indicating that some depression in the detection was caused by the processing of the plasma sample. The preparation of a standard curve for corticosterone using corticosterone added to steroid-free plasma and processed in paral-

lel with samples confirmed that a depression of the curve results since
the slope of this standard curve was less than that of other curves.
Using the steroid-free plasma standard curve has the advantage that no
recovery correction or correction for aliquots is necessary since all
standards and samples are subject to the same losses.

The standard deviation of replicate determinations of cortisol
in the same plasma sample where the concentration of cortisol was in the
range 9 - 22 μg/100 ml was 2.65. This is comparable to the results of
Murphy (106), Nugent (108) and Bowman (109) using competitive protein
binding radioassays. The fluorometric methods of Stewart (31), Van der
Vies (40), and Vermeulen (139) have similar standard deviations although
Martin (34) and Nielsen (37) claim better precision in a similar range
of cortisol concentration. Table XXXV shows precision and normal values
determined by several methods.

Normal morning concentrations of cortisol were found to be
11.71 μg/100 ml. For corticosterone using 5% human plasma, ^{3}H-cortisol
and 60 mg Fuller's earth the morning concentration was found to be 4.96
μg/100 ml; while using 1% human plasma, ^{3}H-corticosterone and 8 mg of
Florisil with steroid-free plasma standards, the level of corticosterone
was determined as 1.84 μg/100 ml. For cortisol, the value compares
favourably with the values by more specific double isotope dilution
methods and also to values determined by fluorometric and CPB methods
(Table XXXV). The value for corticosterone, using 5% plasma, seems high
compared to other reported values, although Van der Vies using a fluoro-
metric method, reported a similar value. Both the present method and
the Van der Vies method employed carbon tetrachloride/water partitioning
to separate cortisol and corticosterone. Possibly some common interfer-
ence affected both values.

The occurrence of negative values for corticosterone may have been due to the presence of protein in the sample. This may have bound the corticosterone and made it unavailable to displace radioactive steroid from the CBG isotope solution. Murphy (106) had found negative values in the assay of cortisol in cerebrospinal fluid and attributed these results to the presence of protein. Complete deproteinization in our studies did not alleviate the problem. The presence of chromic acid residues on the glassware may be sufficient to affect the small quantities of steroid (140) and could explain the negative values. The problem was not due to an adsorbent effect since both Florisil and Fuller's earth gave negative values no matter whether ^{3}H-corticosterone or ^{3}H-cortisol were used to label the CBG.

The competitive protein binding radioassay and the fluorometric assay used in this investigation may be compared by reference to the standard curves. The range of the fluorometric assay is greater (0.005 - 1 µg/ml compared to 0 - 40 mµg for the CPB method). The sensitivity of the CPB method is better. The CPB method is probably more reliable since it is less susceptible to interference by non-steroid material. Impurities in the sample for fluorometry could be charred by the sulfuric acid resulting in decreased detection of fluorescence through absorption of the fluorescent light by this coloured material. Quenching of fluorescence can result from non-steroid material in the final sample. Many materials (eg. dust, bits of rubber) in the final sample could give rise to spurious fluorescence giving anomalously high readings. There was some evidence of interference (probably non-steroid) in the CPB assay of corticosterone. The precision of the CPB method appears to be better, although the much larger range of the fluorometric standard curve contributed to some extent to its increased variability.

CONCLUSIONS

1. Separation of cortisol and corticosterone can be achieved using carbon tetrachloride/water partitioning with one backwash of each phase; however separation is not complete. The carbon tetrachloride phase will contain several steroids other than corticosterone in concentrations which may be significant relative to the corticosterone concentration.

2. The individual determination of cortisol and corticosterone by fluorometry based on the different relative fluorescence in various strengths of sulfuric acid (39) does not give reliable results in samples containing amounts of cortisol and corticosterone in varied proportions. This failure was ascribed to the finding that the ratio of fluorescence of cortisol/corticosterone varied with concentration of the steroid.

3. Competitive protein binding radioassay using Fuller's earth as adsorbent to separate free and bound steroid and using human plasma as the source of corticosteroid binding globulin does not provide a system specific for cortisol. Corticosterone was found to interfere to an extent of greater than 1 mμg/mμg. Specificity for corticosterone relative to cortisol was not achieved using either Florisil or Fuller's earth as adsorbent and human plasma as CBG source. Cortisol was detected in these systems to approximately equal extents.

4. Carbon tetrachloride/water partitioning provided adequate separation of corticosterone and cortisol so that plasma cortisol could be determined by competitive protein binding radioassay in the presence of five to ten times the normal corticosterone concentration without interference from corticosterone. Plasma corticosterone could not be determined by CPB radioassay subsequent to carbon tetrachloride/

water partitioning.

5. When the competitive protein binding radioassay and fluorometric assay are compared as to precision and sensitivity, the CPB method is found to be superior. The fluorometric method has the advantage of wider range.

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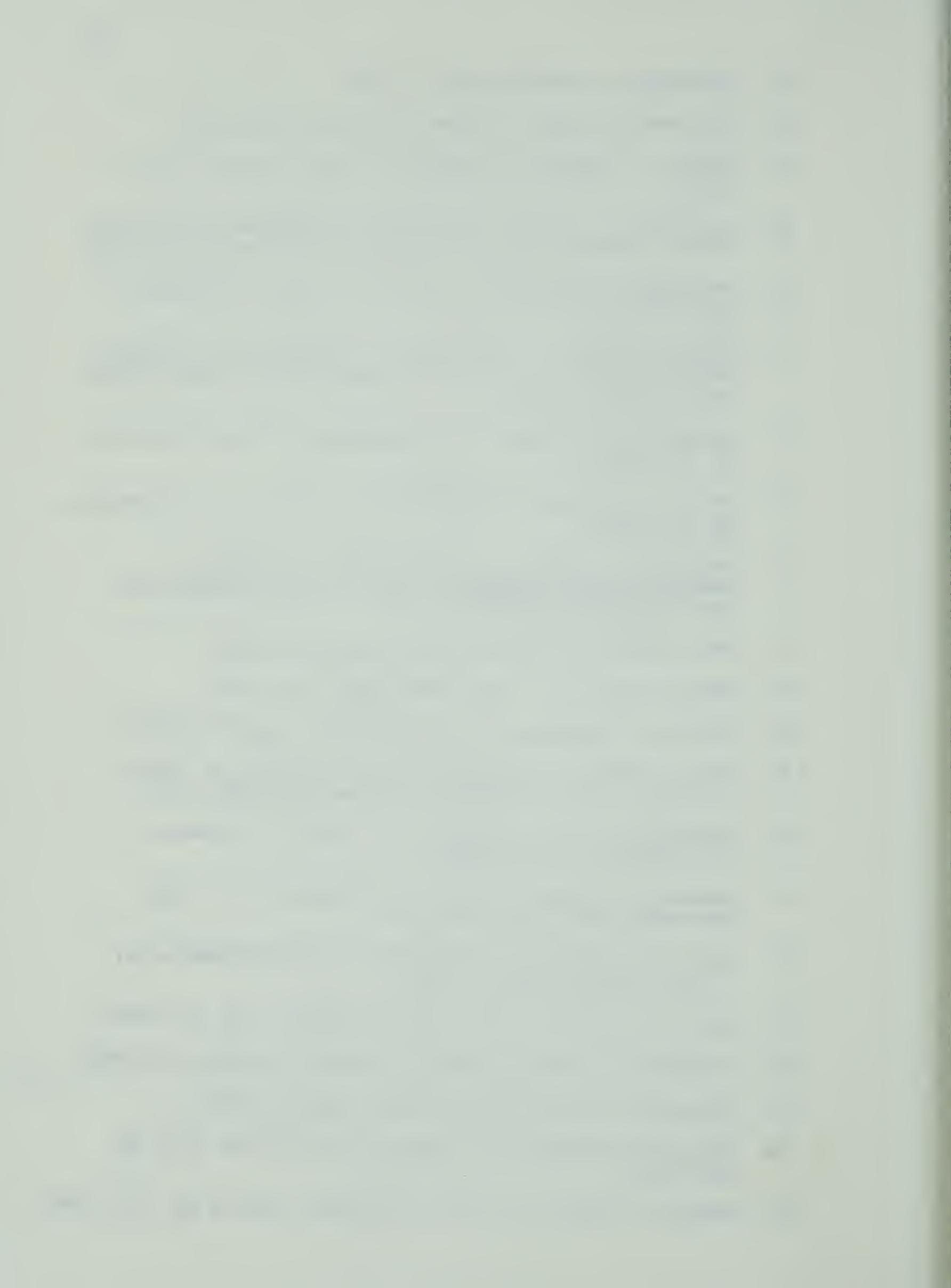
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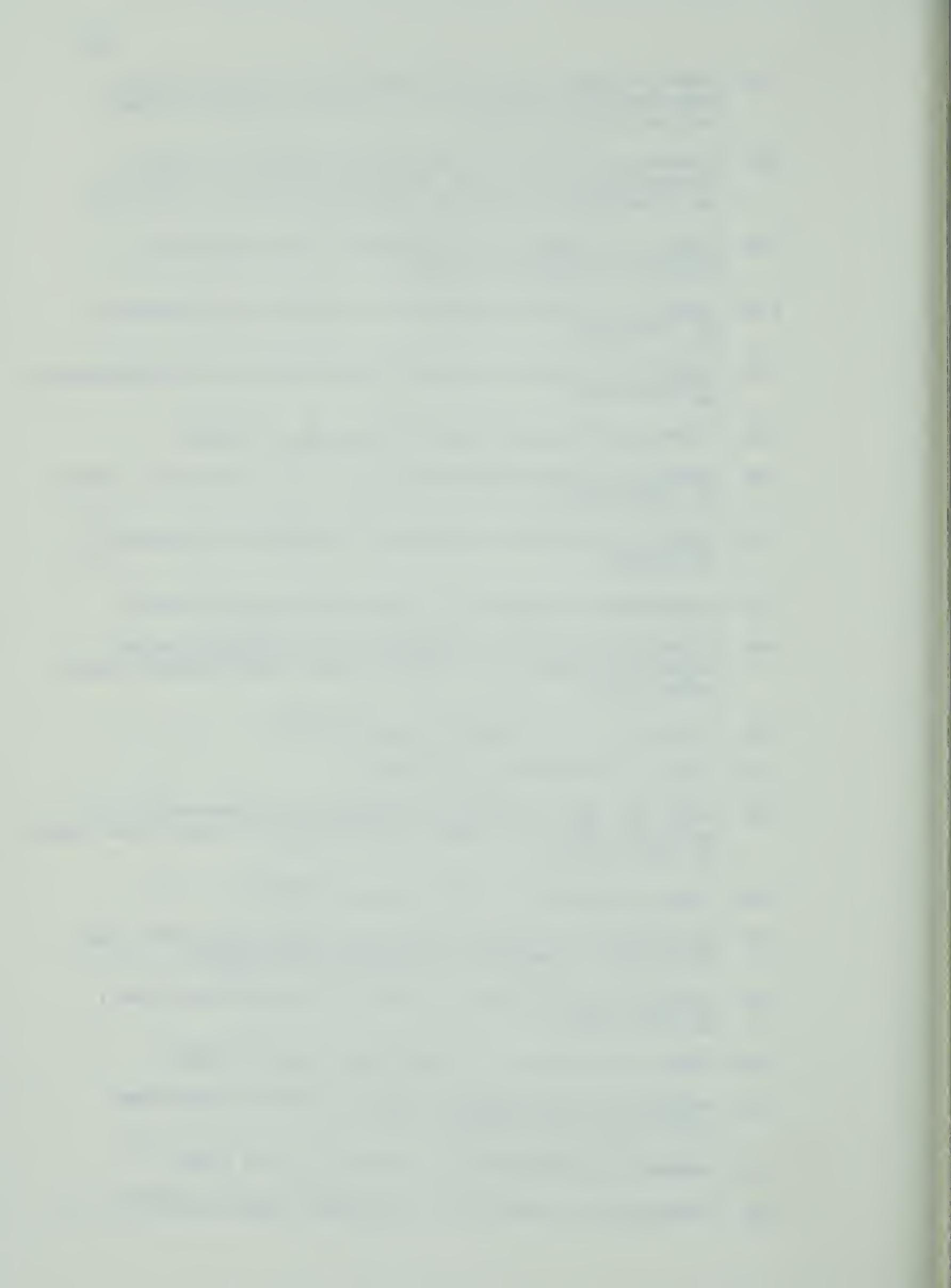
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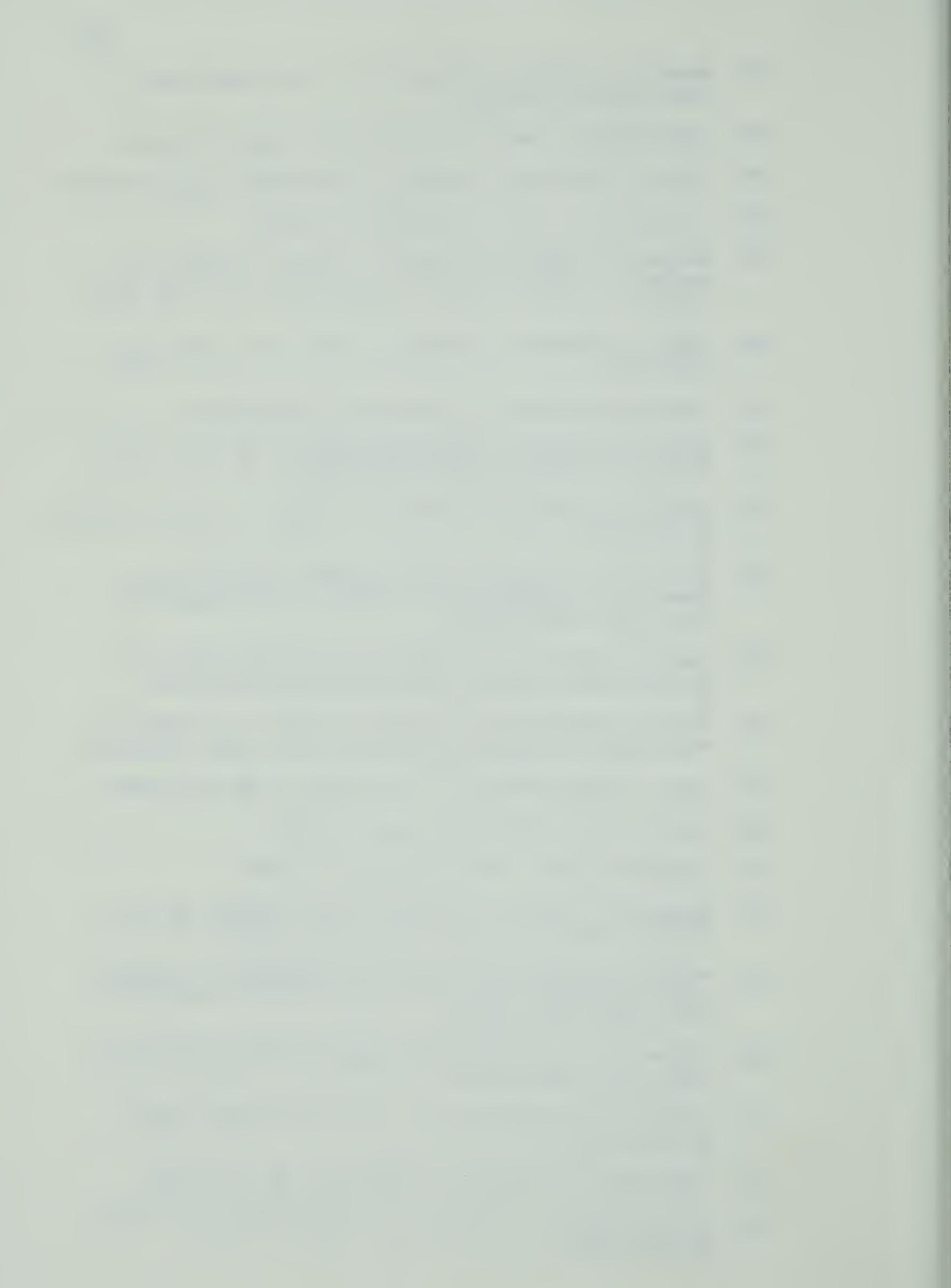
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APPENDIX

TABLE XXIX.

Thin Layer Chromatography Systems for Steroids.

Refer-ence	System	Aldo	B	Chol	E	F	P	T	S	BAc	EAc	FAc	Other
	SILICA GEL												
(141)	Cf												
(142)	EA[a]			0.42	0.38	0.38	0.34		0.37		0.64		DOC 0.56
													DOCA 0.71
(143)	Cf:t-Bu (9:1)	0.39[b]	0.18[b]			0[b]							
		0.39[c]	0.18[c]			0.66[c]							
			0.30[d]			0.77[d]							
(143)	Cf:E (90:1)	0.58[b]	0.40[b]			0[b]							
		0.58[c]	0.40[c]			0.79[c]							
			0.51[d]			0.91[d]							
(144)	Cf:abs E (19:1)	0.07	0.15		0.13	0.07			0.22	0.44	0.38	0.39	AldoAc 0.34
(145)	Cf:E (19:1)	0.14	0.24		0.19	0.08			0.31				DOC 0.60
													DOCA 0.70
(144)	Cf:abs E (9:1)	0.28	0.31		0.34	0.18	0.93	0.87	0.41				AldoAc 0.59
(141)	EA:Cf (50:1)	0.20	0.47	0.89	0.56	0.45	0.62	0.37	0.70				DOC 0.72
(146)	EA:C (1:1)		0.07			0.07			0.20	0.25		0.24	SAc 0.48
													TAc 0.75
(147)	EA:C (1:1)	0.84	0.11		0.07	0.06	0.59		0.50				
(144)	EA:C (1:1)									0.11	0.13	0.14	SAc 0.28
(142)	EA:H (1:1)[a]				0.07	0.06	0.36		0.15		0.18		DOC 0.18
													DOCA 0.37
(144)	B:abs E (9:1)	0.16	0.16		0.17	0.12			0.23				
(148)	B:E (17:3)[e]				0.23	0.20		0.42					
(144)	B:abs E (4:1)	0.31	0.38		0.43	0.37			0.49				AldoAc 0.48
(149)	B:M (9:1)									0.46		0.34	AldoDiAc 0.56
(144)	Bu sat with W		0.38[b]		0.23[b]	0.25[b]			0.25[b]				
(150)	PE:isoBu (1:1)				0.51	0.51					0.63	0.68	

TABLE XXIX (continued)

Refer-ence	System	Aldo	B	Chol	E	F	P	T	S	BAc	EAc	FAc	Other
	SILICA GEL												
(108)	Mc:E (22:3)		1.41[f]		1.45[f]	1.00[f]							
(142)	Cf:M:W (188:12:1)		0.27		0.22	0.13	0.79		0.33				DOC 0.62
(143)	Cf/M:Am (19:1/4:1)	0.17[b]	0.11[b]			0.09[b]							
		0.17[c]	0.11[c]			1.00[c]							
			0.19[d]			1.00[d]							
(143)	Cf/M:Am (19:1/3:1)	0.37[b]	0.22[b]			0.25[b]							
		0.37[c]	0.22[c]			1.00[c]							
			0.33[d]			1.00[d]							
(150)	Cf:PE:Bu (5:5:1)		0.19		0.20	0.07							
(142)	EA:Cf:W (90:10:1)	0.16	0.22		0.28	0.22	0.61		0.35		0.57	0.43	DOC 0.51
(144)	EA:C:abs E (9:9:2)	0.13			0.31	0.28			0.38				
(144)	EA:H:gla HA (15:4:1)	0.41[b]	0.25[b]		0.29	0.26			0.39				
(143)	C:BA:E (9:9:2)	0.41[c]	0.25[c]			0.48[c]							
			0.26[d]			0.44[d]							
						0[b]							
(141)	B:A:W (75:50:0.2)	0.49	0.72[b]	0.94	0.71	0.65	0.96						DOC 0.92
(143)	Mc:EA:E (15:4:1)	0.30[b]	0.27[b]			0[b]							
		0.30[c]	0.27[c]			0.57[c]							
			0.34[d]			0.74[d]							
(143)	Mc:B:M (3:1:1)	0.70[b]	0.68[b]			0.21[b]							
		0.70[c]	0.68[c]			0.74[c]							
			0.66[d]			0.85[d]							
(141)	Mc:M:W (150:9:0.5)	0.40	0.60	0.86	0.48	0.26	0.93	0.79	0.60		0.77	0.70	DOC 0.92
(150)	Cf:PE:isoBu:B (4:2:1:1)				0.41	0.18							
(150)	Cf:PE:isoBu:Ct												
	(25:9:9:10)		0.25		0.30	0.13			0.46		0.63	0.51	
	(10:4:3:3)		0.23		0.33	0.19			0.41		0.77	0.62	
	(5:3:3:3)				0.35	0.17					0.68	0.58	
	(2:1:1:1)				0.38	0.15					0.67	0.59	

TABLE XXIX (continued)

112.

Reference	System	Aldo	B	Chol	E	F	P	T	S	BAc	EAc	FAc	Other
(144)	EA:H:gla HA:abs E (72:13.5:10:4.5)	0.26	0.37		0.47	0.44			0.57				
(141) (151)	Mc:M:W:G (150:10:1:0.4)	0.50	0.75	0.91	0.64	0.38	0.93	0.87	0.73				DOC 0.90
	sequential chromatography												
	Cf:E (99:1)												
	EA:EC:W (90:10:1)												
	Cf:M:W (94:6:0.5)	0.41	0.68		0.72	0.61							
	KEISELGUHR												
(152)	Cf:M:HA (18:1:1)		0.47		0.36	0.2			0.57				
(153)	Partition tlc Formamide as stationary phase												
	Cf				0.71	0.39							
	Cf:T (1:3)										0.59	0.41	
	Mc:T (3:2)				0.33	0.11					0.77	0.59	
	Mc:T (1:1)				0.23	0.10					0.72	0.53	
	CELITE												
(142)	Partition tlc Formamide as stationary phase												
	Cf				0.78	0.40			0.94				
	Cf:B (1:1)				0.30	0.13			0.77				
	POLYAMIDE												
(154)	Cf:A (4:1)				0.66	0.52							
	Mc:A (4:1)				0.70	0.52							

(The "P T S" columns are grouped under the heading "Rf of Steroids".)

TABLE XXIX (continued)

a   Starch bound silica.
b   Periodate oxidation product.
c   Bismuthate oxidation product.
d   Chromate oxidation product.
e   Silica gel:alumina 95:5.
f   Mobility relative to $F = 1$.

TABLE XXX.

Paper Chromatography Systems for Steroids.

Reference	System	Rf of Steroids											
		Aldo	B	DOC	E	F	P	T	S	BAc[a]	EAc[a]	FAc[a]	Other
(155)	T:L:M:W (4:6:7:3)[b]									0.75[c]	0.60[c]	0.45[c]	
	T:L:M:W (4:6:9:1)[b]										0.30[c]		
(156)	Reverse phase pc												
	Mes:M:W (3:2:1)[b]												AldoDiAc 0.47
(157)	Adsorption chromatography on alumina papers (ascending)												
	B:Cf (3:1)						0.75	0.40			0.05	0.02	DOCA 0.70
	B:Cf (2:1)						0.95	0.60		0.15	0.10	0.06	DOCA 0.85
	B:Cf (1:1)						1.00			0.67	0.40	0.30	
	B:A (19:1)						0.80			0.20	0.10	0.05	DOCA 0.70
	Partition chromatography[b]												
A.	LP:M:W (5:4:1)			0.39	0.01	0	0.85	0.40			0.04		SAc[a] 0.25 / DOCA 0.70
B1.	T:LP:M:W (5:5:7:3)		0.23	0.86	0.05	0.03			0.27		0.39	0.25	
B2.	T:LP:M:W (67:33:60:40)		0.65	0.95	0.20	0.10			0.70				
B3.	T:LP:M:W (67:33:80:20)		0.15	0.75	0.02	0.01			0.20				
B4.	T:M:W (2:1:1)		0.70		0.30	0.15			0.72				
B5.	B:M:W (2:1:1)		0.85	1.00	0.50	0.32			0.87				
C.	T:EA:M:W (9:1:5:5)				0.70	0.40							
(158)	LP:M:W (20:17:3)						0.56	0.10					
	LP:B:M:W (67:33:80:20)		0.037	0.478	0.005		0.844	0.424	0.056	0.349	0.122		
	T:M:W (3:2:1)	0.232	0.606	0.882	0.290	0.156		0.839	0.618				
	LP:B:gl.HA:W (67:33:85:15)		0.066	0.258	0.015	0.012	0.591	0.335		0.156	0.051		DOCA 0.461 / SAc 0.181
(159)	Ascending chromatography on glass fibre paper												
	B:DMF (100:0.5)		0.41			0.11			0.66				

TABLE XXX (continued)

Reference	System	Aldo	B	DOC	E	F	P	T	S	BAc[a]	EAc[a]	FAc[a]	Other
(160) B5.	B:M:W (2:1:1)	0.26	0.86	1.00	0.43	0.25			0.86	1.00	1.00	0.91	DOCA 1.00 / SAc 1.00
NB1.	I:T:M:W (5:5:7:3)		0.08	0.60	0.013	0.009			0.091	0.52	0.16	0.085	DOCA 0.95 / SAc 0.54
NB2.	I:T:M:W (33:67:60:40)		0.47	1.00	0.08	0.031			0.50				
E1.	I:M:W (10:9:1)		0.004	0.87	0	0			0.007	0.031	0.007	0.004	DOCA 0.24
E2B.	I:t-Bu:W (10:5:9)	0.36	0.79	1.00	0.59	0.50			0.91	1.00	1.00	0.93	DOCA 1.00 / SAc 1.00
E4.	I:t-Bu:M:W (20:9:9:2)		0.091	0.28	0.04	0.029			0.13	0.23	0.12	0.14	DOCA 0.29 / SAc 0.49
E5.	IPE:t-Bu:W (8:5:6)		0.18	0.72	0.038	0.033			0.31	0.52	0.27	0.30	DOCA 1.00
E9.	H:t-Bu:M:W (20:9:9:8)	0.15	0.40	0.70	0.23	0.18			0.49				
(161)	Cf:F[d] B:F[d] T:Pg[d] B:Pg[d]	no Rf values because solvents overrun paper.											

a 21-acetate.
b Stationary phase is underlined. Each phase is equilibrated with the other.
c Pipsyl esters rather than acetates.
d Paper is impregnated with stationary phase by dipping in 50% methanol solution, blotting excess.

TABLE XXXI.

Methods for Determining 17-Hydroxycorticosteroids using Porter-Silber Reagent.

Refer-ence	Sample	Sample Work-up	Color Development	Range Sensitivity	Recovery
(162)	plasma 15 ml	-ext 3x with CHCl$_3$, evap -Florisil cc, elute 17-OHCS with 24.5% EtOH/CHCl$_3$, evap -dissolve in MeOH, add P-S reagent, cover tube with glass ball	-heat 1 hr at 60°C -read at 410 mμ	0.6 - 4 μg[a]	67 - 93% (F)
(20)	plasma 10 ml	-ppt proteins with 95% EtOH, filter wash 2x with Skellysolve B, evap (incubate with glucuronidase if det'n of conjugates desired) ext 3x with CH$_2$Cl$_2$ -Florisil cc, elute CS with 25% EtOH/CH$_2$Cl$_2$, evap -add EtOH and P-S reagent	-incubate 16 hrs at room temp -read at 370, 410, 450 mμ in micro-cuvettes (Allen corr)	<1 μg	
(163)	plasma 0.5 ml	-ext with CH$_2$Cl$_2$, wash with 0.2 N NaOH, dry with anhyd Na$_2$SO$_4$, evap -partition between H$_2$O/C$_6$H$_6$, back-wash C$_6$H$_6$ with H$_2$O, combine H$_2$O ext with CH$_2$Cl$_2$, dry with Na$_2$SO$_4$[b] evap -dissolve in CH$_2$Cl$_2$, ext with P-S reagent	-incubate 12-20 hrs at 20°C or 30 min at 60°C -read at 370, 390, 410, 430, 450 mμ in 0.03 ml micro-cuvettes (Allen corr)	10 - 120 μg %	62 ± 3% (from H$_2$O)

TABLE XXXI (continued)

Reference	Sample	Sample Work-up	Color Development	Range Sensitivity	Recovery
(164)	plasma 7.5 ml	-ppt proteins with 50% EtOH & ZnSO$_4$ then add abs EtOH, evap supernatant -partition between CHCl$_3$/H$_2$O, wash CHCl$_3$ with 0.1 N NaOH, dry with Na$_2$SO$_4$[b] -ext 'free 17-OHCS' into P-S reagent -hydrolyze H$_2$O with glucuronidase repeat partitioning -ext 'conj 17-OHCS' into P-S reagent (17-KS stay in CHCl$_3$ when ext'd with P-S and may be determined with Zimmerman reagent)	-incubate $\frac{1}{2}$ hr at 60°C -read at 370, 410, 450 mμ in 0.5 ml micro-cuvettes (Allen corr)	1 - 6 μg[a]	90 - 97% (conj 17-OHCS)
(159)	plasma 5-10 ml	-ext 3x with CHCl$_3$, wash with 0.1 N NaOH, evap -silica gel cc, elute with CHCl$_3$: acetone (1:1) -pc on glass fibre paper -determine F & S with P-S reagent -determine B fluorometrically	-incubate overnight at 25°C -read at 370, 410, 450 mμ		72% ± 9 (F) 79% ± 9 (B) 77% ±10 (S)
(165)	plasma 10 ml	-ext 3x with EtOAc, evap -solvolysis & hydrolysis with glucuronidase -silica gel cc to separate 17-KS & 17-OHCS -determine 17-KS with Zimmerman reagent, determine 17-OHCS with P-S reagent.	-incubate 18 hr at 25°C -read at 370, 410, 450 mμ (Allen corr)	5 μg%	80%

118.

TABLE XXXI (continued)

Refer-ence	Sample	Sample Work-up	Color Development	Range Sensitivity	Recovery
(166)		-modified from (22) -evap CH₂Cl₂ to dryness or to 0.5 ml -add P-S reagent (both F & S are re-covered)	-read at 370, 390, 410, 430, 450 mμ		
(167)	plasma 10 ml	-add 6 μg F to plasma, ext with CHCl₃ wash 2x with 0.1 N NaOH, divide in two portions -ext one with blank & one with P-S reagent	-incubate 30 min at 60°C or overnight at room temp -read at 410 mμ		90% (F)
(168)	plasma 10 ml	-ext 3x with CHCl₃, evap -Florisil cc to separate 17-KS & 17-OHCS -ext 17-OHCS fraction with P-S reagent	-incubate 1 hr at 60°C -read every 2 mμ from 360 - 440 in micro-cuvettes	1 - 8 μg^a (E)	
(169)	plasma 20 ml	-ext 3x with CHCl₃, evap -dissolve in 70% MeOH, wash 3x with toluene/hexane, 2x with pentane, evap dissolve in EtOAc, wash with 5% Na₂CO₃ then 0.5 N HCl sat with NaCl, evap -dissolve in MeOH, add 60% H₂SO₄, incubate 5 min at 60°C, cool, read every 10 mμ from 370-460 -add P-S reagent & incubate 20 min at 60°C, repeat readings			93% (80-104) (E)

TABLE XXXI (continued)

Reference	Sample	Sample Work-up	Color Development	Range Sensitivity	Recovery
(170) (procedure for free 17-OHCS)	plasma 10 ml	-add MeOH, shake, wash with CCl_4, acidify, refrigerate, wash 2x with hexane, add abs EtOH & aq BaOAc, refrigerate, collect supernatant evap, dissolve in H_2O, (hydrolyze with glucuronidase or acid to determine glucuronide or sulfate conjugates) ext with n-BuOH, mix, refrigerate, add anhyd Na_2CO_3, shake centrifuge, decant ext, split in 2 evap -dissolve in EtOH, add CH_2Cl_2, ext with blank or F-S reagent	-incubate 18 hrs at room temp -read at 370, 410, 450 mμ semimicrocuvettes (1.1 ml)		88 - 93% (free 17-OHCS) 79 - 90% (conj 17-OHCS)
(171)	plasma 10 ml	-ext 3x with $CHCl_3$, evap -Florisil or Celite cc -dissolve in 0.2 ml MeOH, add 0.3 ml P-S reagent	-incubate 1 hr at 60°C cool -read at 410 mμ in microcuvettes (0.5 ml)	2 - 10 μg[a]	55 - 107% (Florisil) 83 - 112% (Celite)
(22)	plasma 5 ml	-ext with CH_2Cl_2, wash with 0.1 N NaOH, split ext in 2 -ext into P-S or blank reagent	-incubate 10 min at 50° C, 3.5 hr at 37°C, or 8 - 24 hrs at room temp -read at 410 mμ	4 μg%	95.5%
(172) Total or (conj & free) 17-OHCS	plasma or serum 5 ml	-ppt proteins with 10% $ZnSO_4$, add 0.5 N NaOH, shake, centrifuge, collect supernatant, acidify with 50% H_2SO_4 ext with n-BuOH, add Na_2CO_3, shake stand, centrifuge, evap -add blank reagent, incubate 20 min at 60°C, cool, read at 410 mμ, add P-S reagent, reincubate & repeat reading		2 - 8 μg[a]	65%

TABLE XXXI (continued)

Reference	Sample	Sample Work-up	Color Development	Range Sensitivity	Recovery
(27)	plasma 5 ml	-ext with CHCl$_3$, wash with NaOH -ext into P-S reagent	-incubate 30 min at 60°C -read at 370, 410, 450 mμ (Allen corr)	20 - 100 μg%	92% (F)
(173)	plasma 10 ml	-add 5 μg F, ext with CHCl$_3$, wash with 0.1 N NaOH, split in 2 -ext with blank or P-S reagent	-incubate overnight at room temp or 30 min at 60°C -read at 410 mμ (0.8 ml microcuvettes)		
(21)	plasma 12 ml or more	-wash with CCl$_4$, then pet ether, ext with CH$_2$Cl$_2$, freeze aq layer, re-centrifuge, repeat, wash with 0.1 N NaOH, split in 2 -ext into blank or P-S reagent	-incubate overnight -read at 410 mμ	0.5 - 3 μg/ml[a]	92%
(174)	plasma 2.4 ml	-same as (21)	-read in special microcuvettes (0.07 ml)		
(175)	plasma 5 - 10 ml	-ext 3x with CHCl$_3$, evap -Florisil cc, elute 17-OHCS with 15% MeOH/CHCl$_3$, silica gel cc, elute S with 1% MeOH/CHCl$_3$, elute F with 5% MeOH, evap -add P-S reagent	-read at 370, 410, 450 mμ	1 - 10 μg[a]	70% ± 5

TABLE XXXI (continued)

Refer-ence	Sample	Sample Work-up	Color Development	Range Sensitivity	Recovery
(23)	plasma 10 ml	-ext with CH$_2$Cl$_2$, wash with 0.1 N NaOH, split in 2 -ext with blank or P-S reagent	-incubate 16 - 18 hrs at room temp or 30 min at 60°C -read at 410 mμ		91.5 - 102%

a Range of standard curve.
b Anhydrous.

TABLE XXXII.

Fluorometric Methods for the Determination of Corticosteroids.

Reference	Steroid	Sample	Sample Work-up	Fluorescence Reagent & Development	Activation & Emission Wavelength (mμ)	Range or Sensitivity	Recovery
(176)	F & B[a]	serum or plasma 10 ml	-add N NaOH to pH 9 ext 2x with EtOAc wash with H_2O, evap -silica gel cc on -partition cc on Celite to separate F & B, evap -add fluor reagent	-60% H_2SO_4/ EtOH -Incubate 20 min at 45°C (B) 30 min at 450 C (F)	470 530	0.025-0.1 μg[b] (B) 0.15-0.37 μg[b] (F)	80% (F) 72% (B)
(177)	F & B	plasma 20-50 μl brought to 100 μl with H_2O	-wash with iso-octane ext with $CHCl_3$, wash with 0.1 N NaOH -ext into fluor reagent	-65% H_2SO_4/abs EtOH -Incubate 45 min	462 518	0.01-0.08 μg[b] (B) 0.02-0.06 μg[b] (F)	100% (B)
(178)	B	plasma 30-40 ml	-add ^{14}C-B, ext with CH_2Cl_2:CCl_4 (1:1) wash with 0.01 N NaOH, 0.02 N HOAc then H_2O, evap -pc C:B:M:W (4:4:2:1) evap eluate -dissolve in CH_2Cl_2 count one portion ext other with fluor reagent	-65% H_2SO_4/ EtOH -Incubate 2-4 hrs	470 540	0.2 μg%[c]	100%
(179)	F & B	plasma or urine 5 ml	-wash with pet ether ext with CH_2Cl_2, wash with 0.1 N NaOH -ext into fluor reagent	-70% H_2SO_4/ EtOH	520 570		

TABLE XXXII (continued)

Reference	Steroid	Sample	Sample Work-up	Fluorescence Reagent & Development	Activation & Emission Wavelength (mμ)	Range or Sensitivity	Recovery
(180)	11-OHCS (F & B)	plasma 2 ml	-ext with CH_2Cl_2 -ext into fluor reagent	-70% H_2SO_4/ EtOH -Incubate 13 min	475 530	0.1-40 μg^b (F)	82-106% (F)
(181)	B (if F absent from sample	plasma 1 ml	-same as (177)	-70% H_2SO_4/ EtOH -Incubate 30-40 min	470 530		106.2%
(182)	F & B[a]	plasma 5 ml	-wash with pet ether dilute with H_2O, ext with CH_2Cl_2 -wash with 0.1 N NaOH dry with anhyd Na_2SO_4 t.l.c on cellulose (heptanol sat with H_2O) -elute with MeOH, add fluor reagent	-70% H_2SO_4/ EtOH -Incubate 30 min	436 G-4 filter	0.02 μg (F) 0.008 μg (B)	
(183)	F	plasma 0.1 ml tissue 30-70 mg	-wash 2x with pet benzin ext with CH_2Cl_2, wash with 0.1 N NaOH, dry with $MgSO_4^d$, evap under N_2 at 45°C -partition between H_2O and CCl_4, wash H_2O with CCl_4, ext with CH_2Cl_2 -ext into fluor reagent	-70% H_2SO_4/ EtOH -Incubate 25 min -read in 0.07 ml micro-cuvettes	468 524	0-20 mμg^b	100.2 ± 11.7 (s) %

TABLE XXXII (continued)

Reference	Steroid	Sample	Sample Work-up	Fluorescence Reagent & Development	Activation & Emission Wavelength (mµ)	Range or Sensitivity	Recovery
(108)	F	plasma 5 ml + ^{14}C-F	-dilute with N NaOH ext with CH_2Cl_2, wash with H_2O, evap in air -tlc Mc:E(88:12) elute with acetone, count aliquot, evap remainder dissolve in CH_2Cl_2 -ext into fluor reagent	-70% H_2SO_4/ abs EtOH -Incubate 30 min	470 525		68 ± 7.1 (s) %
(184)	11-OHCS	plasma 1 ml	-same as (179)	-70% H_2SO_4/ EtOH -Incubate 15 sec with intense shaking	400-70 520-40	1.5 µg%[c]	103.8%
(185)	CS	plasma 5 ml	-same as (179)	-70% H_2SO_4/ EtOH -Incubate 30 min at 22°C	436 520-70		
(40)	F & B[a]	plasma 2.5 ml	-wash with pet ether dilute with H_2O, ext 2x with CH_2Cl_2, wash with 0.1 N NaOH, dry with Na_2SO_4, evap -partition between H_2O/ CCl_4, wash CCl_4 with H_2O (B in CCl_4), wash initial H_2O with CCl_4 (F in H_2O) -ext into fluor reagent (B directly from CCl_4; F from CH_2Cl_2 ext of H_2O)	-70% H_2SO_4/ EtOH -Incubate 1 hr	400-500 >500	0-75 µg% (F) 0-24 µg% (B)	

125.

TABLE XXXII (continued)

Reference	Steroid	Sample	Sample Work-up	Fluorescence Reagent & Development	Activation & Emission Wavelength (mμ)	Range or Sensitivity	Recovery
(139)	F	plasma 2 ml	-same as (40) except did not use CCl₄ to determine B	-70% H₂SO₄/EtOH -Incubate 10 min	470 550	1.2 $\mu g\%^c$	78%
(186)	CS	CSF 6 ml serum 4 ml	-acidify to pH 1, ext 2x with CHCl₃, wash with 0.1 N NaOH, then H₂O, evap -dissolve in H₂O, wash 2x with pet ether or benzene, ext with CHCl₃, dry with Na₂SO₄ -ext into fluor reagent	-70% H₂SO₄/EtOH -Incubate 40 min	470 520		
(187)	F & B	plasma 1-4 ml bring to 4 ml with H₂O	-same as (180)	-75% H₂SO₄/EtOH -Incubate 5-20 min	470 530		
			Note: Author judged method unsatisfactory due to interfering fluorogens				
(137)	11-OHCS	plasma 1-8 ml	-wash with pet ether dilute with H₂O, ext with CH₂Cl₂, wash with 0.1 N NaOH -ext into fluor reagent	-75% H₂SO₄/EtOH -Incubate 5 min (timed)	470 560	1.8 $\mu g\%^c$	101.4%

TABLE XXXII (continued)

Reference	Steroid	Sample	Sample Work-up	Fluorescence Reagent & Development	Activation & Emission Wavelength (mμ)	Range or Sensitivity	Recovery
(188)	F (B fraction fluorescence not stable so not used)	plasma 2 ml	-add 3H-F in 0.9% saline, ext with CH₂Cl₂ -cc to separate F & B -ext aliquot with fluor reagent -count other aliquot	-77.5% H₂SO₄/ EtOH -Incubate 30 min	Hg source (Chance ON-20 filter) 545		78.3 ± 4.7 (s) %
(189)	F & B[a]	plasma 3 ml	-ext with CH₂Cl₂, wash with 0.1 N NaOH, then H₂O, evap aliquot -silica gel cc to separate F & B, evap -add fluor reagent	-75% H₂SO₄/ EtOH -Incubate 30 min	480 525		88% (F) 90% (B)
(190)	F & B[a]	plasma	-ext with CH₂Cl₂, wash with 0.1 N NaOH, then H₂O, evap -ext B with CCl₄, wash with pet ether-EtOH -F obtained by difference (F = Total CS-B)	-75% H₂SO₄/ abs EtOH	470 530 or 438 524		

TABLE XXXII (continued)

Reference	Steroid	Sample	Sample Work-up	Fluorescence Reagent & Development	Activation & Emission Wavelength (mμ)	Range or Sensitivity	Recovery
(34)	F & B[a]	plasma 2 ml + 0.2 ml N NaOH	-ext with CH_2Cl_2, divide in half, acidify, evap -one part serves as blank to which is added EtOH hydrox-ylamine reagent, stand for 1 hr, dilute with H_2O, treat as below from hexane step on -other portion, add EtOH reagent, stand 1 hr, dilute with H_2O, wash with hexane, ext with CCl_4 (contains B), ext aq EtOH phase with CH_2Cl_2 (contains F) -ext all blank and sample CCl_4 and CH_2Cl_2 phases with fluor reagent	-10% EtOH in 85% aqueous H_2SO_4 -Incubate 1 hr	470 520	1 μg% (F)[c] 0.4 μg% (B)[c]	>80% (F, B)
(39)	F & B[a]	plasma 8 ml (dog) less required for man	-ext 2x with $CHCl_3$, wash with 0.1 N NaOH, then H_2O; dry with Na_2SO_4 evap -add Girard P reagent react 30 min at 37.5°C stop reaction with Na_2CO_3 wash with ether (removes Chol) hydrolyze hydrazones with 6 N HCl, wash with pet ether ext with $CHCl_3$, wash with H_2O, dry with Na_2SO_4, evap -add fluor reagent	-60 & 75% H_2SO_4/EtOH -Incubate 20 min at 45°C, cool 5 min in ice	470 530	0.1 μg%[c]	70-84% (F) 83-90% (B)

TABLE XXXII (continued)

Reference	Steroid	Sample	Sample Work-up	Fluorescence Reagent & Development	Activation & Emission Wavelength (mμ)	Range or Sensitivity	Recovery
(37)	F & B	plasma 2 ml	-ext with CH_2Cl_2, wash with 0.1 N NaOH -ext into fluor reagent	-75% H_2SO_4/EtOH -Incubate 5 min	470 540		100% (F)
(191)	11-OHCS	plasma 1 ml	-wash with hexanone ext with CH_2Cl_2, wash with Na_2SO_4 sol'n then H_2O -ext into fluor reagent	-75% H_2SO_4/EtOH -Incubate 1 hr		0.03 μg (F) 0.01 μg (B)	
(192)	F & B[a]	blood	-wash with pet ether ext 5x with CH_2Cl_2 wash with 0.1 N NaOH then H_2O, dry with Na_2SO_4 d -ext into fluor reagent	-75% H_2SO_4/EtOH -Incubate 5 min (F) 30 min (B)	470 530	0-8.75 μg%	
(193)	F & B	serum 1 ml	-same as (177)	-75% H_2SO_4 in redistilled EtOH -Incubate 15 min	467 525	0.05-0.4 μg[b] (F, B)	95.5-105.4% (F)
(31)	F & B[a]	plasma 1.2 ml	-wash with pet ether add 0.05 N NaOH, ext 2x with CH_2Cl_2, evap at 30°C in vacuo -add 0.2 ml EtOH, 1.8 ml fluor reagent	-75% H_2SO_4/ H_2O -Read at 2, 45 then every 5 min till 120 min, bg only 2 min, F max at 45, B max at 85			97% (F) (79-116) 88% (B) (70-100)

TABLE XXXII (continued)

Reference	Steroid	Sample	Sample Work-up	Fluorescence Reagent & Development	Activation & Emission Wavelength (mμ)	Range or Sensitivity	Recovery
(44)	CS	plasma 1 ml	-wash with iso-octane dilute with H_2O, ext with CH_2Cl_2, wash with 0.1 N NaOH -ext into fluor reagent	-80% H_2SO_4 -Incubate 30-90 min	470 530	0.1-0.5 μg[b]	100% (B)
(42)	CS by semi-automatic procedure	plasma 2 ml	-dilute with 2.5 N NaOH ext with CH_2Cl_2 -remainder of procedure automatic	-80% H_2SO_4/ EtOH -Incubate $3\frac{1}{2}$ min	468, 472 481 mμ from a Zn source Chance OY4 filter	1 μg%[c]	94% (F) (80-110)
(194)	F	plasma 2 ml	-ext with $CHCl_3$ -tlc	-80% H_2SO_4/ EtOH -Incubate 3 ± $\frac{1}{2}$ min	480 530		
(136) extended method	B	plasma 2 ml	-add 3H-B, ext 3x with CCl_4:CH_2Cl_2 (1:1), ext with CH_2Cl_2, wash with ice cold 0.1 N NaOH, 0.2 N HOAc, H_2O, evap -partition between 13% EtOH/Skellysolve B, discard Skellysolve, ext with CH_2Cl_2, evap -pc Table XXX (157), evap dissolve in H_2O, ext with $CHCl_3$, count aliquot -ext other portion with fluor reagent	-48 ml H_2SO_4 + 20 ml 50% EtOH -Incubate 2 hr	filter transmitting above 391 540	1.2 μg%[c]	91.3 ± 0.12%

TABLE XXXII (continued)

Reference	Steroid	Sample	Sample Work-up	Fluorescence Reagent & Development	Activation & Emission Wavelength (mμ)	Range or Sensitivity	Recovery
(195)	F & B[a]	plasma 5 ml	-ext 3x with EtOAc, evap -Florisil cc, silica gel cc, evap -dissolve in MeOH, add H2SO4	-90% H2SO4/MeOH (F) -83% H2SO4/MeOH (B) -Incubate 3 min	480 540	0.1-1 μg^b	50-70%
(196)	F & B[a]	plasma 5 ml	-ext 2x with EtOAc wash with N NaOH, 1% HOAc, then evap -pc B5 Table XXX (157) -add H2SO4 to EtOH eluate	-94% H2SO4/EtOH -Incubate 30 min		0.1-1 μg^b	92% (F) (86-96) 87% (B) (82-96)

a Steroids determined individually.
b Range of standard curve.
c Limit of quantitation in terms of plasma concentration.
d Anhydrous.

TABLE XXXIII.

Double Isotope Analysis of Corticosteroids.

Reference	Steroid	Indicator Reagent	Sample	Purification	Sensitivity[a]
(155)	F	F-Pipsyl ester-131I Pipsan-35S[b]	peripheral plasma 5-10 ml	-ext with CHCl3, evap, dissolve in 70% MeOH/H2O, wash with 20% toluene/hexane evap, dissolve in EtOAc, wash 2x with H2O, dry with Na2SO4[b], evap -esterify:dissolve in 7% pyridine/CHCl3 at 0°C, add pipsan-35S & more solvent react 5 min, stop with H2O -add indicator, wash 2x with H2O, then 50% MeOH/H2O -purify:pc Table XXX (155) -count (GM)	4 μg%
(197)	F, E, B, S	Steroid Pipsyl ester-125I 60 mCi/meq Pipsan-35S 120 mCi/meq	peripheral plasma 10 ml	-ext (155) except ext 2x -esterify:same as (155) except temp -20°C -add indicator, wash with 25% MeOH/H2O, evap -pc #1 T:L:M:W (3:7:6:4) -pc #2 T/F for F, E; T:L (8:2)/F for S, B -count (GM & crystal counter) to 10,000 counts	
(130)	F, B	Steroid Pipsyl ester-125I or 131I Pipsan-35S 45 mCi/mM	peripheral plasma 5 ml	-ext 3x with CHCl3, evap, dissolve in 70% MeOH/H2O, wash with 20% toluene/hexane evap, dissolve in CHCl3, wash 2x with 5% Na2CO3, 3x with H2O -esterify:-18°C, Pipsan-35S, pyridine catalyst, react 5 min, stop with H2O -add indicator in CHCl3, wash with 0.1 N H2SO4, then H2O -pc #1 reverse phase ethyloleate/HA:W (1:1) -pc #2 L:T (1:4)/F, oxidize ester of B -pc #3 L:T (1:1)/F -count (GM or crystal)	50 μg% (F) 60 μg% (B) using 125I

TABLE XXXIII (continued)

Refer- ence	Steroid	Indicator Reagent	Sample	Purification	Sensitivity[a]
(198)	Aldo	Aldo DiAc-14C 1-5 mCi/mM Acetic Anhydride-3H 400 mCi/mM	adrenal vein plasma 2-3 ml	-ext with CH_2Cl_2, wash with 0.1 N NaOH 0.1 N HOAc, H_2O, evap, dry -acetylate: add pyridine[b] & 3H-AcO2, react 24 hr at 37°C -add indicator, H_2O, ext with CCl_4, wash with H_2O, evap -pc #1 C:B:M:W (4:2:4:1) -pc #2 C:D:M:W (4:4:2:1) -chromium trioxide oxidation -pc #3 C:B:M:W (4:3:4:1) -count (liq scint)	50 mμg%
(73)	Aldo, F, B	Steroid Acetate-14C Acetic Anhydride-3H 400 mCi/mM	adrenal vein blood	-hemolyze with H_2O, ext with cold CH_2Cl_2 wash with 0.1 N NaOH, H_2O -acetylate: 36 hr at 37°C -pc #1 E_4 Table XXX (160) -pc #2 B_3 Table XXX (157) for Aldo, B #3 (198) for F -oxidize Aldo, B -pc #3 NB Table XXX (160) for Aldo E_4 Table XXX (160) for B Ct:M:W (4:4:1) for F -oxidize F -pc #4 B_3 Table XXX (157) for Aldo #3 (198) for B Ct:M:W (4:4:1) for F -count (liq scint) 30 min	

TABLE XXXIII (continued)

Refer-ence	Steroid	Indicator Reagent	Sample	Purification	Sensitivity[a]
(199)	Aldo	Aldo-1,2-^{3}H 90 μCi/μg Acetic Anhydride-^{14}C 50 mCi/mM	peripheral plasma 20-30 ml	-add indicator, add N NaOH, ext with CH$_2$Cl$_2$, wash with 0.01 N NaOH, 0.1 N HOAc, add EtOH, evap -pc #1 C:D:W (4:4:1) -acetylation:add pyridine ^{14}C-Ac$_2$O to dry sample, react 24 hr at 18-24°C, add 50% EtOH, wash with iso-octane, ext with CCl$_4$, wash with H$_2$O, evap -pc #2 C:B:M:W (5:2:5:1) -pc #3 C:D:M:W (4:3:4:1) -pc #4 reverse phase Mes:M:W (3:2:1) -form benzylhydrazone -pc #5 I:Bu:M:W (10:6:10:3) -count (liq scint)	0.5 mμg%
(156)	Aldo also F & B with additional chromatog-raphy	Aldo-^{14}C 46 mCi/mM Acetic Anhydride-^{3}H 1050 mCi/mM	peripheral plasma 20 ml	-add indicator & 1.25 N NaOH, ext with cold CH$_2$Cl$_2$, wash with 0.1 N HOAc, then 3x with H$_2$O, evap, dissolve in 20% EtOH/H$_2$O, wash with cyclohexane, ext 2x with CH$_2$Cl$_2$, wash 3x with H$_2$O, evap, dry -acetylate:(to monoAc), add pyridine 3H-AcO$_2$, react at room temp 16 hr, evap add EtOH, evap, dissolve in acid buf-fered EtOH/H$_2$O, ext 3x with CH$_2$Cl$_2$ evap -tlc-2D 10% E/B, 30% C/EA -acetylate:(to DiAc) with cold AcO$_2$ -tlc-2D 7% E/B, 30% C/EA -pc C:B:M:W (5:2:5:1), Mes:M:W (3:2:1) -count (liq scint)	1.25 mμg%

TABLE XXXIII (continued)

Reference	Steroid	Indicator Reagent	Sample	Purification	Sensitivity[a]

(200)	Aldo, F, B	Steroid-^{14}C 11-145 μCi/mg
		
		 Acetic Anhydride-^{3}H 400 mCi/mM
			adrenal vein plasma up to 50 ml	-add indicator, ext with cold CH_2Cl_2, wash with 0.05 N NaOH, 0.1 N HOAc, evap, dissolve in EtOH/H_2O, wash with cyclohexane ext with CH_2Cl_2, evap, dry
				 -acetylate:add pyridine, ^{3}H-AcO$_2$, react 16-18 hrs at 37°C, stop with 20% EtOH ext with CCl_4, evap
				 -pc #1 C:B:M:W (5:2:5:1)
				 -pc #2 C:D:M:W (4:4:2:1) for F
				      C:D:M:W (4:3:2:1) for Aldo, B
				 -oxidize Aldo, F
				 -pc #3 C:B:M:W (20:14:20:5) for Aldo, F
				      Mes:M:W for B
				 -count (liq scint) 100 min
					40 mμg%

(201)	Aldo also F & B with additional chromatography	Aldo-^{14}C 50 mCi/mM Acetic Anhydride-^{3}H 500 mCi/mM	peripheral plasma 10-30 ml	-add indicator, ext, acetylate as (200) -pc #1 & #2 as (200) -pc #3 Mes:M:W (3:2:1) -oxidize -pc #4 C:B:M:W (20:14:20:5) -count (liq scint) 20x for 10 min	
(202)	Aldo	Aldo-^{14}C 46 μCi/mM Acetic Anhydride-^{3}H 500 mCi/mM	peripheral plasma 20-30 ml	-add indicator, N NaOH, ext with CH_2Cl_2, wash with 0.01 N NaOH, 0.1 N HOAc, H_2O, shake with Biogel P-10, filter, evap -tlc silica gel 15% T/E -acetylate -pc I:T (7:3)/ethylene glycol, tlc 5% T/E -pc I:t-Bu:W (5:1:5) -pc Mes:M:W (3:2:1) -pc I:B:M:W (3:2:4:1) -count (liq scint)	

TABLE XXXIII (continued)

Reference	Steroid	Indicator Reagent	Sample	Purification	Sensitivity[a]
(149)	Aldo, F, B	Steroid-^{14}C Acetic Anhydride-^{3}H	peripheral plasma 25 ml	-add indicator, ext & acetylate as (198) -tlc 10% M/B -pc as #1 & #2 (198) -oxidize, pc as #2 & #3 (198) -count (liq scint) 5 x 100 min for Aldo 3 x 100 min for B, F	

a Limit of quantitation in terms of plasma concentration.
b Anhydrous.

TABLE XXXIV.

Competitive Protein Binding Radioassays for Corticosteroids.

Reference	Steroid	Isotope	CBG Source	Separation of Bound & Free Steroid	Sample	Sample Work-up	Recovery & Range
(109)	F	$3H-F$ 18.3 Ci/mM	0.2% rabbit alpha globulin aqueous	Fuller's earth 15 mg	plasma 0.01 ml	CH_2Cl_2 ext	- 0-10 mμg
(110)	F	$3H-F$ 55 Ci/mM	5% human plasma phosphate buffer	Dextran coated charcoal (0.5 ml) or Fuller's earth 20 mg	plasma 0.1 ml	ext 2x with CH_2Cl_2	100.2% ± 0.3 2.5-25 mμg
(101)	F, B, E	$14C-F$ 65 μCi/mg	1:8 citrated plasma (3 week old) diluted with physiological saline	dialysis 4-9°C for 40 hr pH 6.7	plasma 1 ml	boil with saline to destroy CBG pc to separate steroids	91.9 ± 13.7 (s) % (F) 0-500 mμg
(105)	CS	$14C-F$ 69 μCi/mg	50% human serum aqueous	Sephadex gel filtration	plasma 1 ml	EtOH ppt protein	93 ± 3.6 (se) % (F) 0-300 mμg
(106)	F	$3H-F$ 22.1 Ci/mM	5% human plasma aqueous	Fuller's earth 15 mg	plasma 1 ml urine 1/10,000 24 hr vol	plasma EtOH ppt protein urine CH_2Cl_2 ext	- 0-40 mμg

TABLE XXXIV (continued)

Reference	Steroid	Isotope	CBG Source	Separation of Bound & Free Steroid	Sample	Sample Work-up	Recovery & Range
(106)	CS	^{3}H-B 23.5 Ci/mM	2.5% dog plasma aqueous	Florisil 40 mg	plasma 0.01 ml CSF 0.1 ml	EtOH ppt protein	- / 0-4 mµg
	S	^{3}H-B 23.5 Ci/mM	2.5% dog plasma aqueous	Florisil 40 mg	plasma 0.05 ml	CCl$_4$ ext	- / 0-8 mµg
	F & 17-OH-P	^{3}H-B 23.5 Ci/mM	2.5% dog plasma aqueous	Florisil 40 mg	plasma 0.3 ml	pet ether ext, tlc	- / 0-16 mµg
	B	^{3}H-B 23.5 Ci/mM	0.25% monkey plasma aqueous	Florisil 40 mg	plasma 0.1 ml	tlc	- / 0-4 mµg
	E	^{3}H-B 23.5 Ci/mM	2.5% chicken plasma aqueous	Florisil 40 mg	plasma 0.1 ml	tlc	- / 0-20 mµg
(107)	F	^{3}H-F 22.1 Ci/mM	5% human plasma aqueous	Fuller's earth 15 mg	urine 1/3,000 24 hr vol	ext 2x with CH$_2$Cl$_2$	96 ± 7 (se) % / 0-40 mµg
(108)	CS	^{14}C-F 42µci/mM	33% human plasma in borate buffer	Dextran coated charcoal (0.7 ml)	plasma 1 ml	CH$_2$Cl$_2$ ext wash with H$_2$O	93.8 ± 3.5 (s) % / 0-500 mµg

TABLE XXXV.

Precision and Normal Values for Human Plasma of Corticoid Estimates
by Various Methods

Reference	s^a	Precision range^a	n	Normal Values $\mu g/100$ ml^b	n	Comments
PORTER-SILBER (17-OHCS)^c						
(20)				8 ± 1		6-10 AM
(163)	0.27	0-15	6	12.8		9 AM
(164)				12.7 ± 0.7 se 9.5 ± 0.9 se		free conjugated
(159)	0.85 0.34 0.32	13-16 2- 3 3- 4	10 11 8	8.6 ± 2.36 2.53 ± 0.94 3.34 ± 1.28	9 15 10	F) S) 9-11 AM B)
(167)				18.8	16	8-9 AM
(169)				6.8 ± 1.4	21	male
(170)				13.2 27.1		free conjugated
(171)				4-10		blood
(23)				15 ± 4.5	50	
(172)				41.2	52	8 AM
(27)	3.44	2-45	10	13.7	11	
(173)				13.3 ± 6.2	16	
(21)				19 ± .7		10 ml sample
FLUOROMETRIC (11-OHCS)^c						
(176)				0.2 9.8		B F
(137)	1.7	10-20	24	21.96 ± 4.76		8-9 AM
(188)	0.55	10-20	6	11 ± 4.4	36	F 10-12 AM
(195)				3 ± 0.2 10.9 ± 0.55	20 20	B F
(182)				0.7-2.9 6-15		B F

TABLE XXXV (continued)

Reference	s^a	Precision range^a	n	Normal Values μg/100 ml^b	n	Comments
(203)				11.71 ± 4.31	48	F male age 16-78
				13.01 ± 4.27	33	F female age 16-69
				12.24 ± 4.34	81	F both sexes all values 7:30 AM
(196)				1.2 (0-6)	9	B) 8-9 AM
				9.2 ± 1.5	30	F)
(190)				2 ± 0.5		B
				11 ± 1		F
(34)	0.21	0-0.9	36	0.6 ± 0.6	19	B) 8-9:30 AM
	0.80	10-19.9	39	9.6 ± 3.0	33	F)
(39)	0.1					B
	0.2					F
(37)	0.2	10-20	33	21.4 ± 5.6	18	7 AM
				13.0 ± 3.3	18	9 AM
(178)				1.1 ± 0.3	20	B
				15	20	F
(194)				18 ± 4.3	30	F
(185)				19.1 ± 3.1	18	
(193)	0.082	15-35	44			
(31)	0.34	1-2	9	0.4 ± 0.2	27	B)9:30 AM
	2.12	10-20	9	9.6 ± 2.7	27	F)
(42)	1					
(40)	2.2		16	5.2		B
	2.1		16	13.8		F
(139)	1.2	10-20	75	14.6 ± 3.7	50	F 8 AM

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(197)				2.5	19	B)
				1.8	23	E)
				10.5	159	F) 8-10 AM
				1.3	17	S)

TABLE XXXV (continued)

Reference	s^a	Precision range[a]	n	Normal Values µg/100 ml[b]	n	Comments
(130)				0.56 ± 0.21	23	B AM
				0.34 ± 0.1	13	B PM
				10.27 ± 3.1	29	F AM
				6.77 ± 3.13	14	F PM
(199)				$6.6 ± 1.2^d$	10	Aldo 8-10 AM upright position
(156)	0.35^d			$6.96 ± 4.05^d$	9	Aldo sitting
(201)	0.28^d		8	$5.8 ± 4.3^d$	25	Aldo
				0.34 ± 0.25	25	B
				11.8 ± 4.3	25	F all values 9-10 AM recumbent
(149)	1.5^d	12		8^d	34	Aldo
	0.03	11		0.66	29	B
	0.19	12		9.8	17	F
(202)				$7.49 ± 4.84^d$	20	Aldo

COMPETITIVE PROTEIN BINDING RADIOASSAY

Reference	s^a	Precision range[a]	n	Normal Values µg/100 ml[b]	n	Comments
(109)	4.9	0-100	167			F
(110)				15.7 ± 4.6	26	F 8 AM
				8.2 ± 2.3	25	F 2 PM
				6.4 ± 2.7		F 8 PM
(101)	2.0	10-20	18	15.2 ± 5.1	12	CS male
				17.6 ± 4.3	12	CS female all values 9 AM
(105)	1.2	10-20	20			
(106)	2.0	10-20	20	8-24		CS)
				6-18		F)
				<2		S) 9 AM
				<4		B)
				<4		E)
(108)	1.2	10-20	12	15		CS 8 AM

TABLE XXXV (continued)

Reference	s^a	Precision range^a	n	Normal Values $\mu g/100\ ml^b$	n	Comments
Present study	2.65	9-22	39	11.71 ± 1.50	3	F AM
	2.52	4- 8	13	1.84	1	B AM

a In μg/100 ml; if standard deviation given for several concentration
   ranges, the standard deviation for normal range is quoted.

b ± standard deviation unless indicated otherwise.

c Normal value for this steroid group unless indicated otherwise.

d mμg/100 ml.